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METABOLITES OF CYATHUS AFRICANUS AND A RELATED FUNGUS

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF CHEMISTRY

EDMONTON, ALBERTA

FALL, 1977

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled

METABOLITES OF CYATHUS AFRICANUS AND A RELATED FUNGUS

submitted by DIRK MARINUS JOHANNES VAN SCHIE

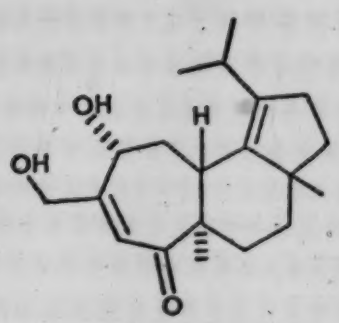
in partial fulfilment of the requirements for the degree of Master of Science

W.A. Ayer, Supervisor

Date 14 October 1977

ABSTRACT

Four metabolites of the bird's nest fungus *Cyathus africanus* were separated by thin-layer chromatography. Two of these were shown to be identical with cyathin A_3 (1) and allocyathin B_3 (2) respectively, both of which were first isolated from *Cyathus helenae*.



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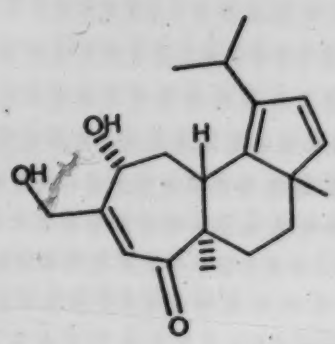

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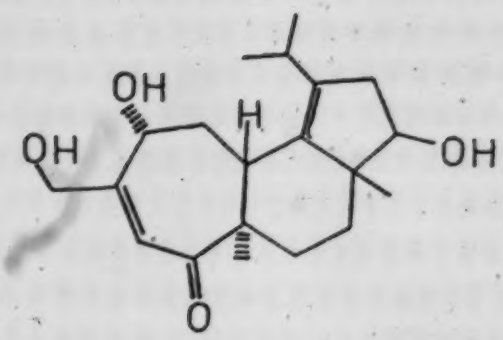
The third metabolite, cyafrin A_4, was shown to possess structure 19 by spectroscopic analysis of the parent compound and its triacetyl and acetonide derivatives. Preliminary work towards the correlation of cyafrin A_4 with cyathin A_3 was undertaken.

The structure of the fourth metabolite, cyafrin B_4 (21), was established by spectroscopic analysis of



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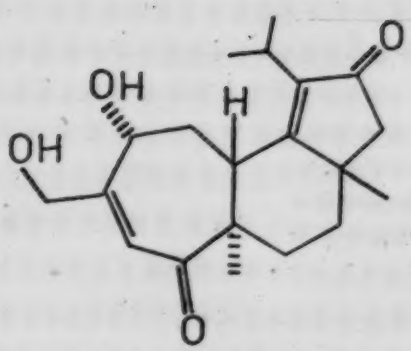

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the parent compound and its diacetyl and methyl ketal derivatives. Many other metabolites were shown to be present but were not isolated.

A preliminary investigation into the metabolites of another bird's nest fungus, Sphaerobulos stellatus, was undertaken.

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I. INTRODUCTION

In recent years a large number of bird's nest fungi have been described[1], most of which have been classified as belonging to the class Basidiomycetes, subclass Homobasidiomycetes, order Gasteromycetes, family Nidulariaceae, genus Cyathus.

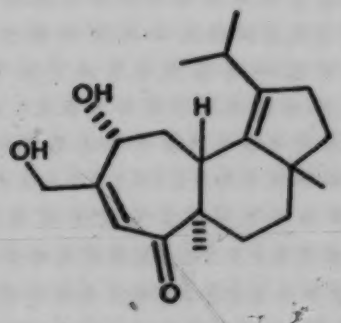
Wilkins[2] reported that mycelium of one of these fungi, Cyathus striatus, showed bacteriostatic properties. This report was confirmed later by Broadbent[3]. In 1967 Olchowecki[4] found that the mycelium of Cyathus helenae also inhibited growth of a number of bacteria. Further studies[1] indicated that mycelium of other species, including Cyathus limbatus and Cyathus poeppigii, also exhibited bacteriostasis.

Of these fungi C. helenae has been studied most extensively. Johri[5] found that an ethyl acetate extract of the liquid medium used for cultivation of this fungus retained its antibacterial activity. The residue from evaporation of this extract was named "cyathin". Johri also carried out studies on the effect of the composition of the nutrient medium and other factors on the growth of C. helenae and the production of cyathin[5,6].

Taube[7] first separated several components of crude cyathin. He identified the phenol 2,4,5-trihydroxy-

benzaldehyde and found a large number of C_{20} compounds, all of which appeared to have the same basic skeleton. These compounds were named cyathins, with a letter and a number added to the name of each to indicate the number of hydrogen and oxygen atoms in the molecule, respectively. Thus compounds of the "A" series contain 30 hydrogen atoms, of the "B" series 28 and of the "C" series 26. The number in each name corresponds to the number of oxygen atoms in the molecule. If a second compound was found with the same molecular formula it was distinguished from the first by the prefix "allo-", and a third one by "neoallo-".

Taube succeeded in obtaining in pure, crystalline form two of these, cyathin A ($C_{20}H_{30}O_3$) (1) and allocyathin B ($C_{20}H_{28}O_3$) (2), and determined their structures by spectroscopic and chemical methods[8,9]. The absolute stereochemistry has been confirmed by X-ray diffraction studies of cyathin A[3].



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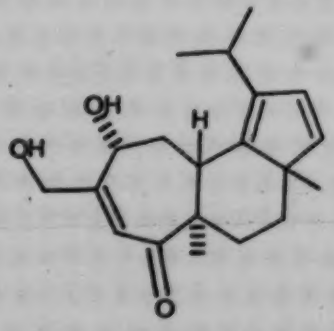

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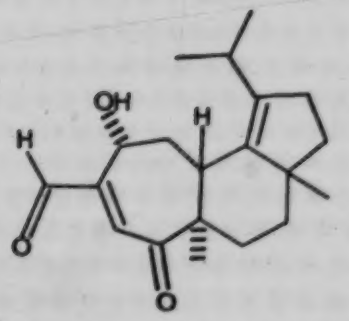
Carstens[10,11] carried on the investigation of crude cyathin. He separated a mixture of cyathin B ($C_{20}H_{28}O_3$) (3) and cyathin C ($C_3C_{20}H_{26}O_3$) (4) from the remaining components of the crude cyathin. He was able to



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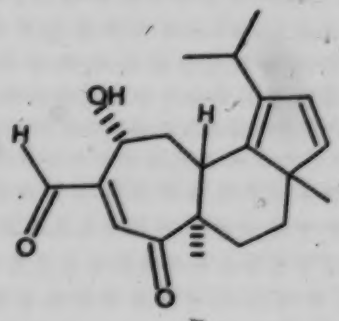

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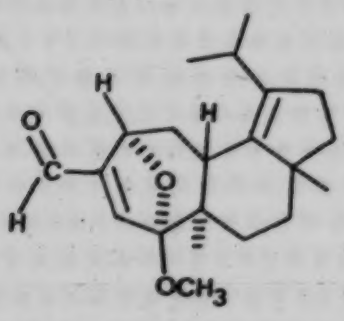
separate them from one another only in the form of their methyl ketal derivatives (5 and 6, respectively). The struc-



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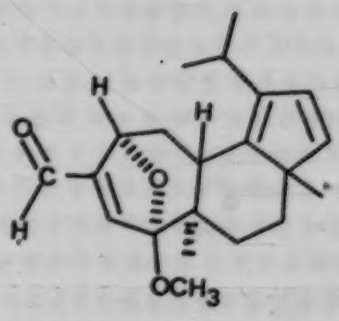

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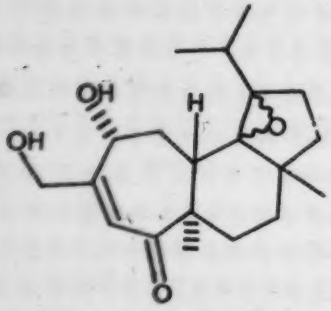
tures of cyathin B_3 (3) and cyathin C_3 (4) were determined from spectroscopic evidence on the methyl ketals (5 and 6 respectively) and confirmed by chemical correlation. The methyl ketal of cyathin B_3 was converted to the methyl ketal of cyathin A_3 and the methyl ketal of cyathin C_3 was converted to the methyl ketal of allocyathin B_3. Later Mercer developed a direct method of separating cyathin B_3 from cyathin C_3[12].

Recent work by Mercer[12] resulted in the isolation of neoallocyathin A ($C_{20}H_{30}O_4$) (7) via its O,O-isopropylidene derivative 8.



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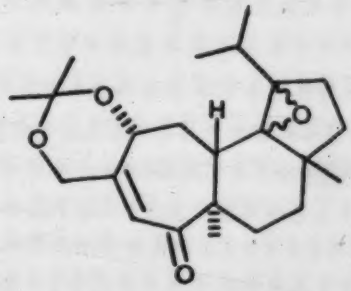

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Two other bird's nest fungi that were subsequently investigated, Cyathus bulleri[13] and Cyathus intermedius[14], were found not to produce any compounds of similar structure to the cyathins.

In 1966 Dr. D. Hocking brought with him from Tanzania two specimens of a bird's nest fungus which he had found growing on dead twigs on Mount Kilimanjaro at an altitude of about 8000 ft. Brodie[15] determined this to be a previously unrecognized species of the genus Cyathus and named it Cyathus africanus.

Johri[5] compared chromatograms of crude cyathin from C. helenae with those of similarly prepared extracts of C. striatus and of the recently discovered Cyathus africanus Brodie, and found them to be very similar, although not identical.

It was the similarity between chromatograms of extracts of C. africanus and C. helenae that prompted the investigations presented in this thesis. To distinguish the metabolites of C. helenae and C. africanus, the crude ethyl acetate extract from C. africanus will be called "cyafrin".

The purpose of this work was thus to determine whether extracts of C. africanus also contain compounds similar to the ones found in C. helenae, and to elucidate the structure of any new compounds isolated.

Another related fungus, Sphaerobolus stellatus, also had been found to exhibit some antibacterial activity. For this reason the metabolites of this fungus were also investigated.

II. GENERAL EXPERIMENTAL

Growth of fungi and preparation of crude extracts

The procedures used for growing both *C. africanus* (strain 66120) and *S. stellatus* are based on those developed for *C. helenae* by Johri[5].

Agar slant tubes of the fungus strain 66120 were obtained from the collection of H. J. Brodie, and were maintained on Brodie solid (agar) medium (for the composition of the Brodie medium used see detailed experimental). These cultures may be stored for long periods on solid medium in slant tubes or on Petri dishes at 5 °C.

The growth of mycelium in liquid medium is initiated by transferring some of the agar-grown mycelium to a 500 ml Erlenmeyer flask containing 250 ml of sterilized Brodie medium. The culture is allowed to grow at room temperature for about four weeks, then the liquid culture is homogenized in a sterilized Waring blender and used to inoculate (by means of a sterile pipette) a number of 2 l Fernbach flasks each containing 500 ml of sterile medium. After about four weeks of growth the liquid is decanted from the flasks. The remaining mycelium may be reflooded with fresh sterilized medium which will yield after a further four weeks a second yield of metabolites. The

quantity of mycelium in the flask has usually increased greatly after the second growth so that repeating the "re-flooding" technique is impractical. A new growth is then initiated.

Crude extracts are prepared by partitioning twice the combined liquid media with half the volume of ethyl acetate. The organic layer is dried with anhydrous sodium sulphate, filtered, and the solvent is removed by means of a rotary evaporator.

Solvents and adsorbants

Because of the small scale on which separations and reactions had to be carried out during this work even small amounts of involatile impurities in solvents could not be tolerated. Therefore all solvents were distilled before use except anhydrous ethyl ether (Mallinckrodt), which was found to be of sufficient purity to be used directly from the container.

The only adsorbent used was Silica gel G (E. Merck). This adsorbent was used directly from the bottle.

Analytical thin-layer chromatography (tlc)

1. Preparation of plates.

Only microscope slides were used, of either

25 x 75 mm or 50 x 75 mm. These were coated with the adsorbent layer with the aid of templates, using a Desaga spreader, according to a method described by Stahl[16]. Routinely a slurry was made up of 50 g silica gel G, approx. 1 g "electronic phosphor" ($ZnSiO_4$, General Electric) and 100 ml H_2O. The spreader was set to give a thickness of adsorbent layer of 0.2 - 0.3 mm. The coated slides were allowed to dry at room temperature for several hours and then activated by heating overnight at 110 - 120 °C.

2. Application of samples and developing of the plates.

Samples were applied as solutions in a solvent as non-polar as possible, using open end melting point tubes which had been drawn out in a flame to give a very fine capillary point. Up to three spots could be applied to a small microscope slide and up to six to a large one.

Plates were developed inside a 250 ml beaker covered with a petri dish. Approximately 10 ml of the appropriate solvent was needed. Development time depended on the solvent system, but was approximately five minutes.

In certain cases the "multiple elution" technique was employed, whereby a plate, after having been developed, was allowed to dry and then returned to the developing tank to be eluted a second time. This process was repeated as often as needed.

3. Detection of components.

Components could be detected either by viewing the plates under uv light (254 nm) or by spraying the plates with a 30% H_2SO_4 solution followed by heating at 110 °C for a few minutes. In the first case spots would show up dark against a bright green fluorescent background, in the second case the spots would be charred to a purple or yellow-brown colour. Some indication as to the relative amounts of material in each spot could be obtained from its size and the intensity of charring.

4. Recording of R_f values and solvent systems used.

R_f values are given as the ratio of the distance moved by the component over that moved by the solvent front. These values are dependent on the solvent system used. This is indicated (by its code letter in parentheses) along with the value. See below for these solvent systems.

In cases where "multiple elution" was applied the R_f was recorded after the final elution. The solvent system was allowed to progress to the same point on each elution.

Solvent systems used were:

A: Benzene : Acetone : Acetic Acid 75 : 25 : 1

B: Chloroform : Methanol 97 : 3

C: Chloroform : Methanol 19 : 1

D: Acetone : Skellysolve B 7 : 3

Preparative thin-layer chromatography

1. Preparation of plates.

For preparative work, plates of size 100 x 20, 20 x 20, 10 x 20 and 5 x 20 cm were used. These were coated in a similar manner to the analytical plates, using a slurry of the same composition, with the spreader set to give a layer of 0.7 - 0.8 mm thickness.

2. Application of samples and developing of plates.

For small samples the application was done by hand, using a small disposable pipette, the tip of which had been drawn out over a flame to a fine capillary, and which was fitted with a rubber bulb over the other end. Solutions were drawn up into the pipette with the rubber bulb and applied to the plate by gently squeezing the bulb while moving the pipette slowly along a line parallel to and about 2 cm from the bottom edge of the plate. For large plates the application was done with a mechanical applicator equipped with a similar capillary.

In general, it was found that 5 mg of material per cm width of plate could be separated without difficulty.

The small plates were developed in glass tanks of about 25 x 25 x 5 cm, which were closed at the top with a glass plate. The large plates were developed in a metal tank (Shandon) of 110 x 25 x 15 cm. Developing time was approximately 50 minutes.

3. Detection of bands and recovery of compounds.

If the compounds involved were strongly uv absorbing, the bands were detected directly by viewing under uv light. If not, detection was done in conjunction with the sulphuric acid "charring" method, used on an analytical chromatogram run on the same sample under similar conditions. First, the corresponding bands as seen under uv light were scored on both analytical and preparative plates, then the analytical plate was sprayed with the 30% sulphuric acid solution and heated at 110 ^{o}C for a few minutes. The location of the desired bands on the preparative plate could thus be inferred from the R_f value of the spots on the analytical plate.

Recovery of the separated compounds was achieved by removing the silica gel in each band from the plate, collecting it in a small glass filter and eluting the compound from the silica gel with a polar solvent such as acetone or methanol. Evaporation of the solvent then left the purified compound.

Measurement and recording of spectra

Mass spectra were recorded on an AEI model MS-2, an AEI model MS-9 or an AEI model MS-50 mass spectrometer. The MS-9 was used to determine molecular formulas by high resolution mass spectrometry (hrms). The MS-50 provided the atomic composition of all fragment peaks in a spectrum. All mass spectra in this thesis are recorded as the intensity of each peak relative to the base peak taken as 100%.

Proton nuclear magnetic resonance (nmr) spectra were recorded on either a Perkin-Elmer R 32 90 MHz spectrometer or a Varian Associates HA-100 100 MHz spectrometer. Spectra of very small samples could be recorded on this latter instrument in the Pulse Fourier Transform mode using the Digilab FTS/NMR 3 Data system. ^{13}C nmr (cmr) spectra were recorded on a Bruker WP-60 instrument. Chemical shifts are given throughout this thesis as δ in ppm, using tetramethylsilane as reference standard. Spin-spin coupling data were obtained by way of double irradiation experiments. Nmr coupling patterns are described as follows: s - singlet, d - doublet, t - triplet, q - quartet, m - unresolved multiplet, u - unresolved absorption band.

Infrared (ir) spectra were recorded on a Perkin-Elmer model 421 dual grating spectrophotometer.

Ultraviolet (uv) spectra were recorded on a Unicam SP 1700 spectrophotometer.

III. RESULTS AND DISCUSSION

Characterization and partial purification of crude cyafrin

During the course of this work portions of crude cyafrin from six consecutive growths were obtained and examined. The amount of crude cyafrin varied between 75 and 150 mg per liter of medium. Crude cyafrin has the appearance of a dark brown, very viscous, oily substance.

Initial experiments by Mr. R. Parker[17] of these laboratories had shown that crude cyafrin could be partially purified by a four funnel counter-current distribution between water and diethyl ether. It is assumed that the result of this procedure is to extract any highly polar substances such as carbohydrates and glycerol into the aqueous phase. Glycerol is one of the constituents of the Brodie medium used for growing C. africanus. The combined ether fractions of the counter-current distribution yielded after evaporation of the solvent a light brown foam. This foam usually constituted about half the weight of the initial oil and was used as the starting material for all following separations.

Later the counter-current distribution was replaced by a simpler procedure which involved partitioning the crude cyafrin between ether and water. Crude cyafrin

was dissolved in the minimum amount of equal volumes of ether and water and the layers separated. The ether was extracted with an equal volume of water, then the two aqueous layers combined and extracted twice with an equal volume of ether. All ether layers were combined and the ether evaporated. Any residual water was removed by freeze-drying. The results of this procedure were as satisfactory as those of the counter-current distribution.

Liquid-solid chromatography over silica gel has proven to be the most effective method of the several separation methods examined for the separation of crude cyathin[9,11,12]. For that reason liquid-solid chromatography was also the first choice for the separation of cyafrin.

Thin-layer chromatography (tlc) was considered more attractive than column chromatography because, once some skill has been acquired in handling and preparing the plates and applying the samples, it is a fast and convenient method and less prone to mechanical problems. Therefore preparative tlc has been the method used throughout this work, both for the initial large scale separations and for later purifications.

Preliminary tests indicated that solvent system A gave the most satisfactory separations of crude cyafrin. Routinely the multiple elution technique was applied; three

elutions were usually sufficient.

It was found that the composition of crude cyafrin varied considerably between different growths. This is not surprising as a similar variability has been found with C. helenae. It is thought that mutations occurring in the mycelium during its growth are responsible for this variation in composition[10,12]. Thus the actual amount of any one metabolite isolated from crude cyafrin could vary from virtually none to as much as 10% of the weight of the crude mixture. Sometimes a certain band on preparative tlc would contain mainly one compound; at other times the same band would contain a complex mixture of compounds. The following results can therefore be considered as representing only a general trend.

In general most of the material was found on the high-polarity (low R_f) side of the chromatograms. Usually a large proportion (40 to 50%) had an R_f of 0.0 (solvent system A, triple elution). This material was not further examined.

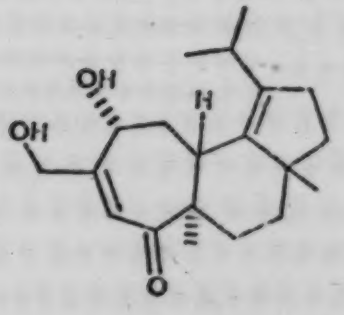
Three components showed up on tlc that appeared to contain substantial amounts of material (as judged from their size and colour-intensity after charring with 30% sulphuric acid). Of these, component A, R_f 0.5 and component B, R_f 0.2 (solvent system A, triple elution) were

coloured intensely purple by the charring while the third, component C, R_f 0.4, charred yellowish brown. These three components were selected for further examination.

Most compounds isolated during the course of this investigation were obtained and handled as more or less colourless gums.

Isolation and identification of cyathin A_3 - allocyathin B_3 mixture

Taube[7,9] had found the major metabolites of C. helenae to be cyathin A_3 and allocyathin B_3 (9 and 10 respectively). He found compounds 9 and 10 to have identical R_f (silica gel, solvent system Benzene : Acetone : Acetic acid 70 : 30 : 1) but he managed to separate 9 and 10 by tlc on silver nitrate impregnated silica gel.



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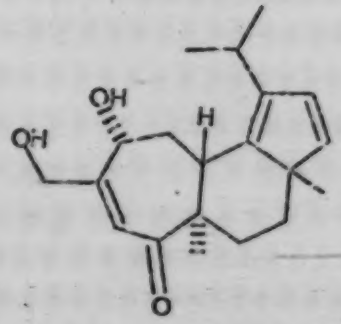

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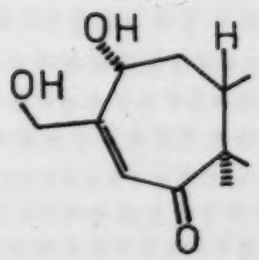
As cyathin A_3 and allocyathin B_3 were the most abundant of all cyathins isolated from C. helenae, it was decided to use them as reference substances for comparison with any compounds found in crude cyafrin.

Cyathin A_3 (sample provided by Dr. P. Singer of these laboratories) has an R_f value on tlc (R_f 0.5, solvent system A, triple elution) identical with that of component A found in C. africanus. Use of other solvent systems such as C (double elution) did not show any differences between component A and cyathin A_3.

The mass spectrum of component A showed four apparent parent ions at m/e 334, 332, 318 and 316. The ions at m/e 318 and 316 were the most intense with an intensity ratio of approximately 3:4. Other prominent ions were found at m/e 191, 189, 175, 45 and 43 (base peak). Determination of the molecular formulas of the parent peaks by high resolution mass spectrometry gave the following results: m/e 316, $C_{20}H_{28}O_3$; m/e 318, $C_{20}H_{30}O_3$, which are the same as the molecular formulas of allocyathin B_3 and cyathin A_3. Thus component A is a mixture of four compounds, two of which are present in major amounts. The molecular formulas of the two minor parent peaks were found to be m/e 332, $C_{20}H_{28}O_4$ and m/e 334, $C_{20}H_{30}O_4$.

The nmr spectrum of component A was very un-

informative, its main features being very broad, completely unresolved bands around δ 1.0, 1.5 and 2.1. In addition it showed unresolved narrower bands near δ 4.5 and 6.0. This behaviour is not unexpected for cyathin-like compounds and has been explained[9] in terms of a fast tautomeric equilibrium between a cyathin and its internal hemi-ketal tautomeric form (Part structures 11 and 12).



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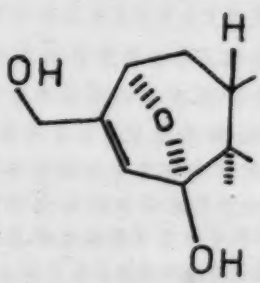

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It was decided to attempt to separate component A by a method originally suggested by Taube[7, p.44]. The procedure involves acetylation of the hydroxyl groups of the compounds in the mixture followed by preparative tlc of the acetylated mixture. If desired, the isolated pure acetylated cyathins can be deacetylated under mild basic conditions in methanol to give the pure cyathins. An additional advantage

of this method is that the tautomeric equilibrium described above is not possible for acetylated cyathins, and it would therefore be expected that these derivatives would give more informative spectral data.

Acetylation of component A was achieved using pyridine and an excess of acetic anhydride overnight at room temperature. After concentration, the acetylated mixture was separated by prep. tlc (solvent system A) and the largest fraction, fraction A (at R_f 0.7) isolated and analyzed.

The mass spectrum of fraction A (see fig. 1) showed parent peaks at m/e 402 (hrms: $C_{24}H_{34}O_5$) and 400 (hrms: $C_{24}H_{32}O_5$) at an intensity ratio of about 3:4. Comparison of the spectrum of fraction A with the mass spectra of 0,0-diacetylcyathin A[7] and 0,0-diacetylallocyathin B[3] measured by Taube[3] shows that fraction A is a 3:4 mixture of these two compounds.

In addition, the nmr spectrum of fraction A in $CDCl_3$ (see fig. 2) can be fully explained if fraction A is assumed to be composed of 0,0-diacetylcyathin A[3] and 0,0-diacetylallocyathin B[3]. Compare tables I and II with fig. 2 (tables also from data published by Taube[7,9]).

It was concluded on this basis that the two

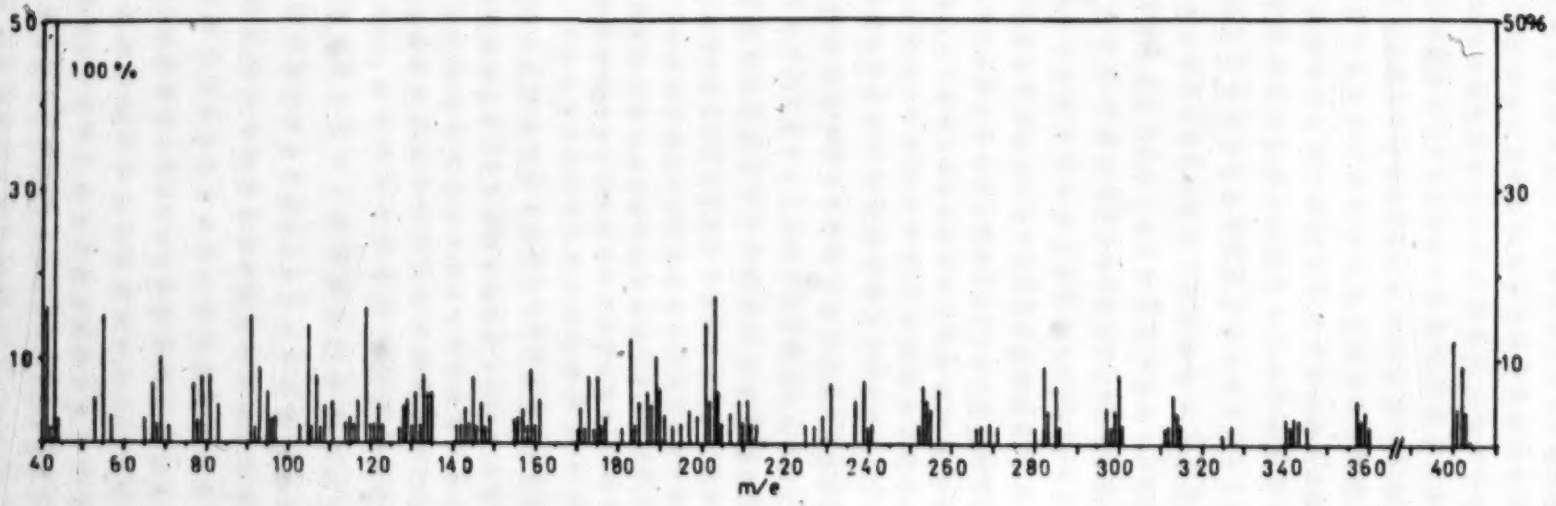


Figure 1. Mass spectrum of fraction A (O,O-diacetylcyathin A_3 and O,O-diacetylallocyathin B_3)

Figure 2. Nmr spectrum of fraction A (O,O-diacetylcyathin A_3 and O,O-diacetylallocyathin B_3) ($CDCl_3$).

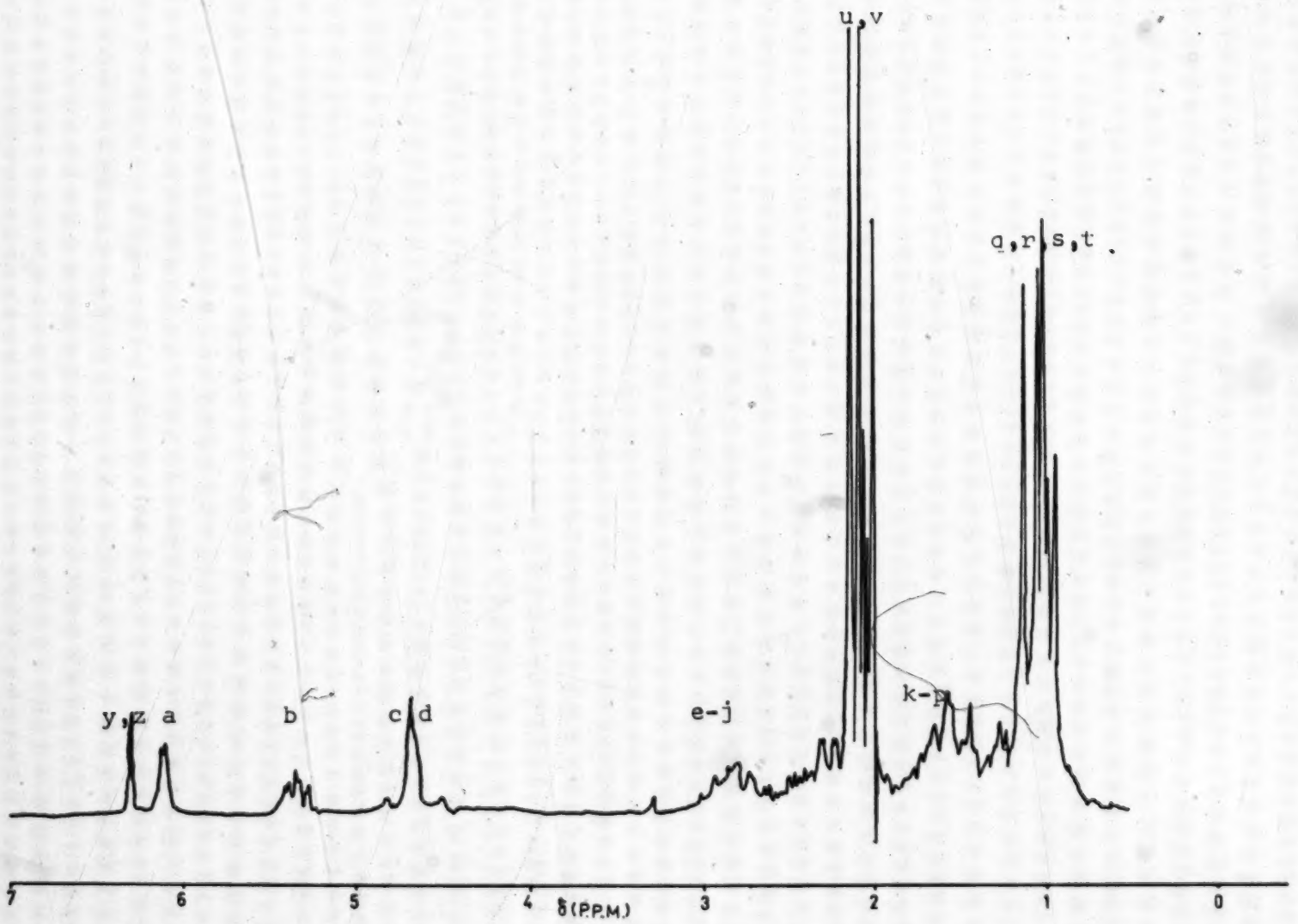

u,v
q,r,s,t
y,z a
b
c d
e-j
k-p
7
6
5
4
δ(P.P.M.)
3
2
1
0

Table I. Nmr data of 0,0-diacetylcyathin A_3 in $CDCl_3$

Signal [a]	Chemical shift (δ)	Number of protons	Multiplicity [b]	Coupling constant (Hz) [c]	Coupled with signal	Assignment
a	6.13	1	d,d,d	1.5,1.5,0.5	c,d,b	13
b	5.36	1	d,d,d,d,d	7,6,0.5,0.5,0.5	j,g,a,c,d	11
c	4.74	1	d,d,d	14,1.5,0.5	d,a,b	15
d	4.63	1	d,d,d	14,1.5,0.5	c,a,b	15
e	2.85	1	q,q	7,7	s,t	18
f	2.78	1	d,d	10,2.5	j,g	5
g	2.53	1	d,d,d	15,6,2.5	j,b,f	10
h,i	2.3 approx.	2	u	?	?	2
j	2.1 approx.	1	d,d,d	15,10,7	g,f,b	10
k-p	1.9-1.1	6	u	?	?	1,7,8
q	1.15	3	s			(16, 17)
r	1.06	3	s			
s	1.00	3	d	7	e	(19, 20)
t	0.99	3	d	7	e	
u	2.12	3	s			(23, 24)
v	2.04	3	s			

a,b,c,d

For footnotes see page 26.

Table II. Nmr data of 0,0-diacetylallocyathin B_3 in $CDCl_3$

Signal [a]	Chemical shift (δ)	Number of protons	Multiplicity [b]	Coupling constant (Hz) [c]	Coupled with
a	6.15	1	d,d	1.5,1.5	c,d
b	5.39	1	d,d	6.5,6.5	g,j
c	4.75	1	d,d	14.5,1.5	d,a
d	4.68	1	d,d	14.5,1.5	c,a
e	2.96	1	q,q	7,7	s,t
f	2.87	1	d,d	10.5,3	j,g
g	2.56	1	d,d,d	14,6.5,3	j,b,f
j	2.27	1	d,d,d	14,10.5,6.5	g,f,b
k,l,m,n	1.9-1.1	4	u	?	?
q	1.04	3	s		
r	1.00	3	s		
s	1.12	3	d	7	
t	1.10	3	d	7	
u	2.12	3	s		
v	2.05	3	s		
y	6.34	1	d	5.5	z
z	6.31	1	d	5.5	y

a,b,c
For footnotes see page 26.

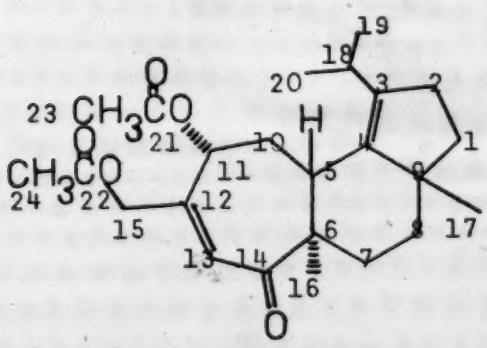
Footnotes to tables I and II.

[a] Identification of signals by letters is as in ref. 7: letters are assigned alphabetically to signals belonging to protons on the basic cyathin structure in order of decreasing chemical shift; remaining letters are assigned to signals belonging to protons added on to the basic structure by derivation. This coding is also used in all following tables of nmr data.

[b] For notation see general experimental.

[c] The constants are listed in the same order as the signals in the following column.

[d] Numbers refer to numbered positions in structure 13



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major compounds present in component A are identical with cyathin A_3 and allocyathin B_3. This work establishes that *C. africanus* does indeed produce metabolites similar to those found in *C. helenae*.

Isolation and proposed structure of cyafrin A_4

The next subject of investigation was component B (R_f 0.2, solvent system A, triple elution). After initial isolation by prep. tlc using this system component B was found to be still contaminated with more polar material. Further purification was achieved by prep. tlc with solvent system D to give pure compound B as judged by tlc. On one occasion a sample was obtained crystalline, but usually compound B was handled as a gum.

The mass spectrum of compound B (see fig. 3) showed a parent peak at m/e 334 and other prominent peaks at m/e 203, 190 (base), 175 and 43. The material appeared to be essentially pure, but a small peak at m/e 332 suggested a minor impurity. Hrms gave a molecular formula of $C_{20}H_{30}O_4$ for m/e 334 and showed the atomic composition of the major fragment peak (m/e 190) to be $C_{14}H_{22}$. By analogy with the general scheme for naming cyathins compound B was called cyafrin A_4.

The nmr spectrum of cyafrin A_4 (see fig. 4)

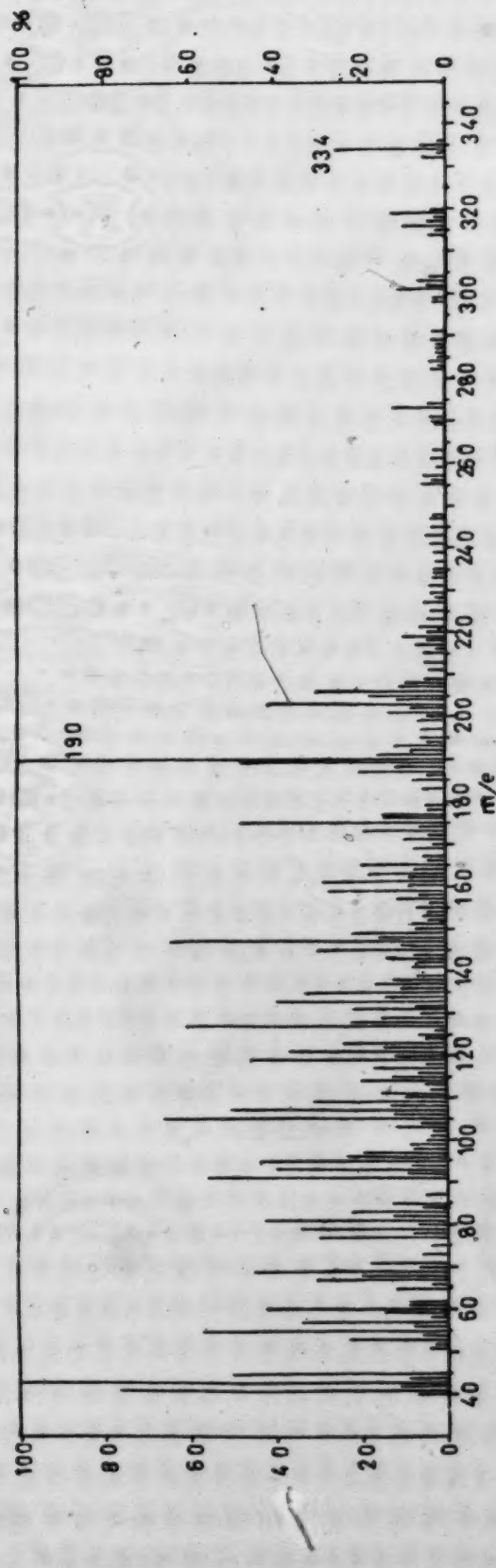
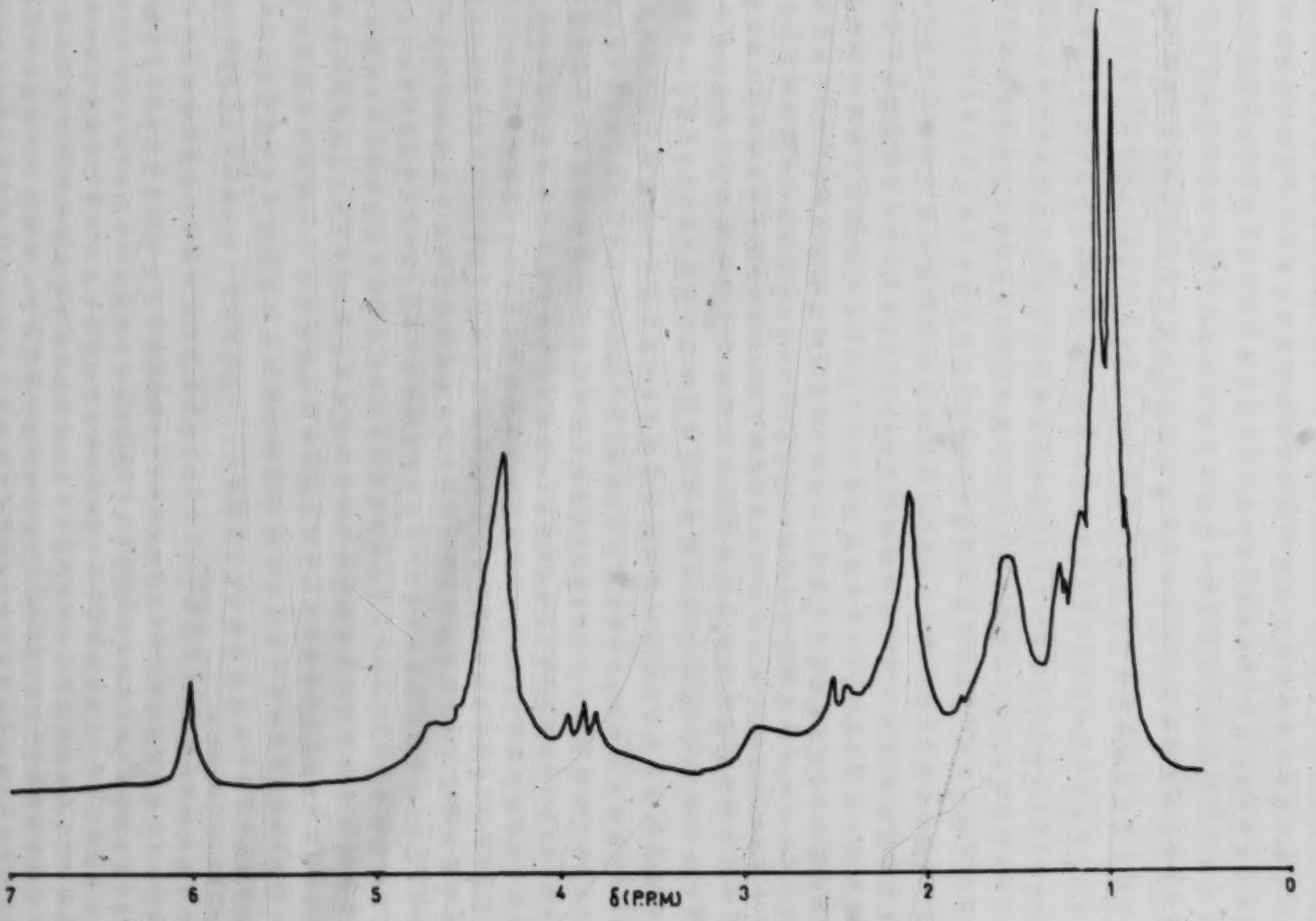


Figure 3. Mass spectrum of cyafrin A_4.

Figure 4. Nmr spectrum of cyafrin A ($CDCl_3$).


7
6
5
4
δ(P.P.M)
3
2
1
0

turned out to be poorly resolved, as anticipated for cyathin-like compounds[7, p.39]. For cyathins the poor resolution in the nmr is attributed to the ketone - hemiketal tautomeric equilibrium described earlier. The only distinguishable feature of the nmr spectrum of cyafrin A_4 that is of interest is the triplet (J = 7.5 Hz) at δ 3.9, which is not present in the nmr spectra of cyathin A_3 and allocyathin B_3.

The infrared spectrum of cyafrin A_4 is shown in fig. 5 (in chloroform solution) and fig. 6 (neat). The second spectrum was obtained by applying a chloroform solution of cyafrin A_4 to the face of the ir cell and allowing the chloroform to evaporate. Both spectra show the general features found in the spectra of cyathins. See for example the spectra of cyathin A_3[7, p.36]. The absorption at 3600cm^{-1} in the solution spectrum may be attributed to free hydroxyl groups. In pure state all hydroxyl groups are hydrogen-bonded, as evidenced by the relatively more intense absorption at 3400 cm^{-1} in the neat spectrum (fig. 6), and the ketone function is completely masked. This phenomenon is also observed in the ir spectra of cyathins and is attributed to the formation of an internal hemiketal. It has been shown that cyathin A_3 crystallizes exclusively in this form[7,9]. The remaining absorption of cyafrin A_4 at 1650 cm^{-1} is possibly due to a number of C=C double bonds in the molecule. The sharp peak at 750 cm^{-1} is due to residual chloroform.

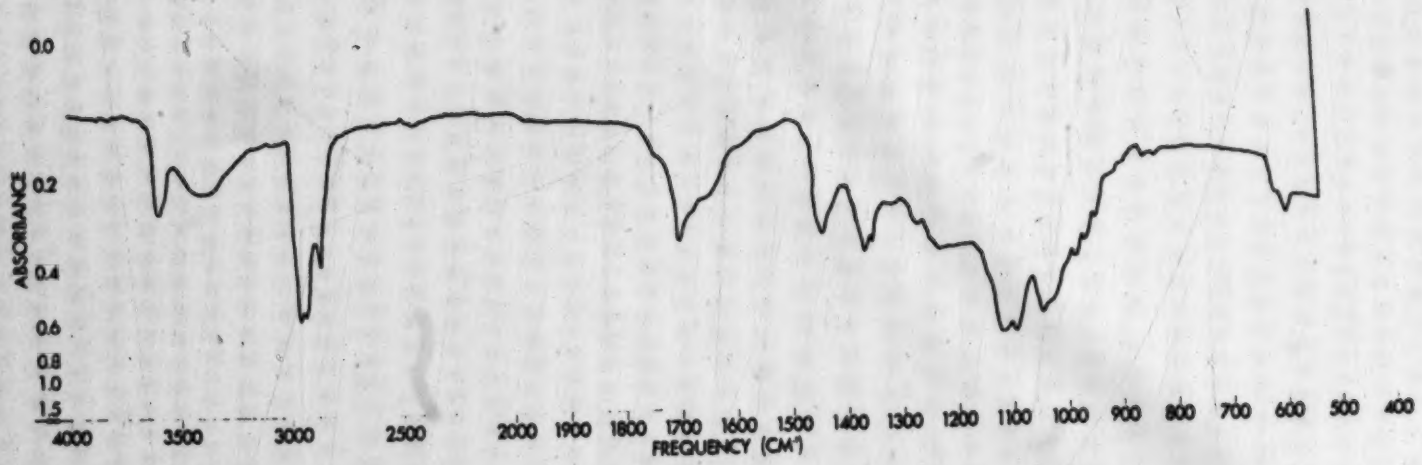


Figure 5. Ir spectrum of cyafrin A_4 ($CHCl_3$ solution).

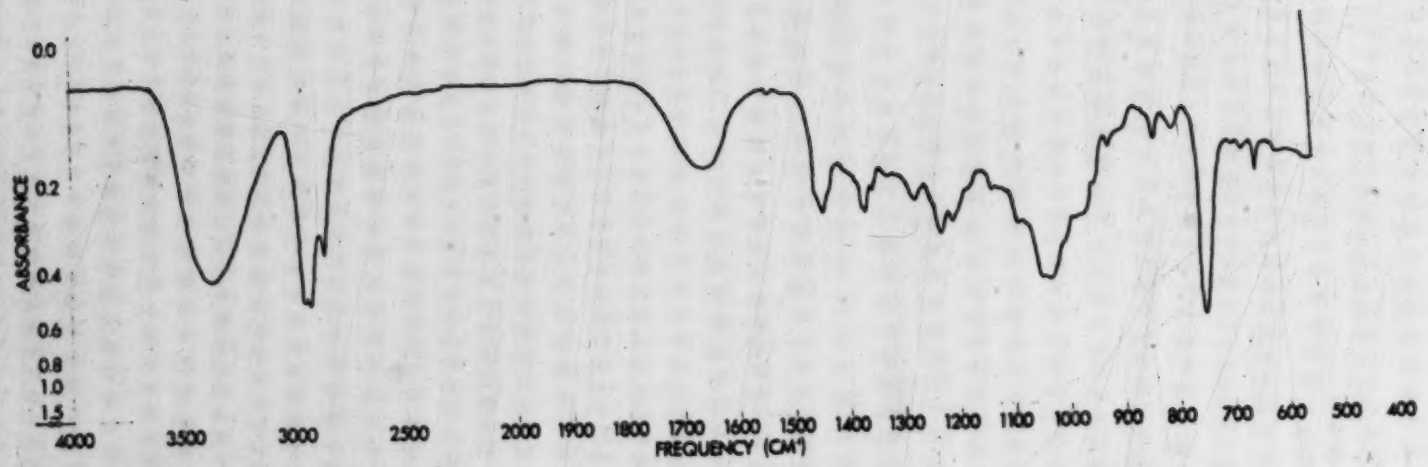


Figure 6. Ir spectrum of cyafrin A_4 (neat).

It thus appears that cyafrin A_4 possesses a cyathin-like structure containing a number of hydroxyl groups. To obtain information on the number of hydroxyl groups in cyafrin A_4 it was acetylated. Acetylation of cyafrin A_4 was achieved in the same manner as described for the cyathin A_3 - allocyathin B_3 mixture, using pyridine as solvent and a large excess of acetic anhydride, overnight at room temperature. The crude product was purified by prep. tlc (solvent system A).

The mass spectrum of acetylated cyafrin A_4 (see fig. 7) shows an apparent parent peak at m/e 400, hrms: $C_{24}H_{32}O_5$. This at first perhaps surprising result may be explained if one assumes the formation of a triacetyl derivative. A triacetyl derivative (which would have a molecular formula of $C_{26}H_{36}O_7$, m/e 460) can eliminate one molecule of acetic acid in the mass spectrometer (high temperature, low pressure) if a proton is suitably positioned on a carbon atom vicinal to that bearing the acetylated hydroxyl group involved. This process would result in the fragment at m/e 400. The small peak at m/e 340 could be the result of the loss of a second molecule of acetic acid. It was thus tentatively assumed that cyafrin A_4 had formed an O,O,O-triacetyl derivative.

This assumption is confirmed by the nmr spec-

trum (see fig. 8). The three sharp singlets at δ 2.02, 2.07 and 2.12 are assigned to the protons of three acetyl groups. The nmr spectrum of 0,0,0-triacetylcyafrin A_4 proved to be very instructive. Many of its features could be analyzed by virtue of its striking similarity to that of 0,0-diacetylcyathin A_3. Compare tables I and III. The data for 0,0,0-triacetylcyafrin A_4 (table III) were obtained using a 100 MHz spectrometer; the information on couplings was obtained by means of spin-spin decoupling (double irradiation) experiments. Table I (0,0-diacetylcyathin A_3) was compiled from data obtained using a 220 MHz spectrometer, which accounts for its greater detail.

A comparison of table I and table III shows only three significant differences. Firstly, 0,0,0-triacetylcyafrin A_4 (table III) shows an extra signal at δ 4.89, which is coupled with some of the protons in the region of signals e to j. Secondly, the region between δ 1.9 and 1.1, which contains six protons in 0,0-diacetylcyathin A_3, contains only four in 0,0,0-triacetylcyafrin A_4. Thirdly, the region around δ 2.1 (signals u,v,w) shows three three-proton singlets in 0,0,0-triacetylcyafrin A_4 instead of two (signals u,v) as observed in 0,0-diacetylcyathin A_3.

The conclusions one can draw from this comparison are: cyafrin A_4 and cyathin A_3 are likely to have

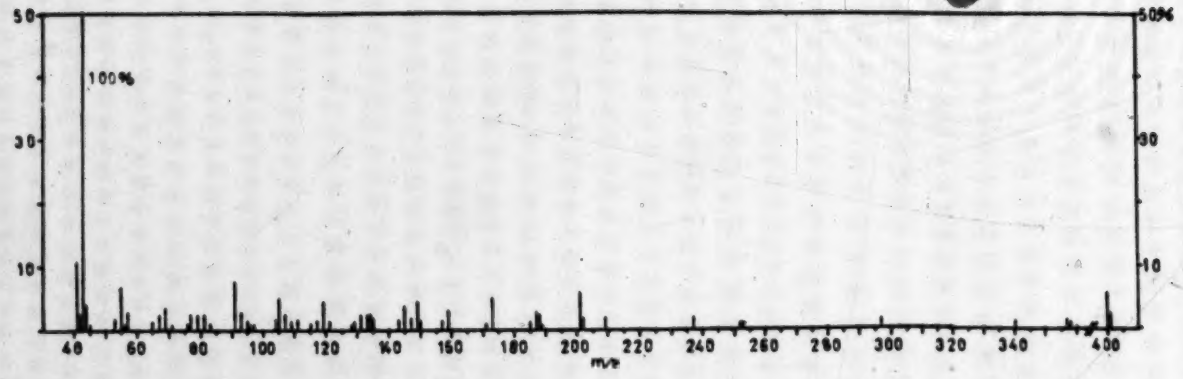


Figure 7. Mass spectrum of 0,0,0-triacetylcyafrin A$_4$

Figure 8. Nmr spectrum of 0,0,0-triacetylcyafrin A_4 ($CDCl_3$).

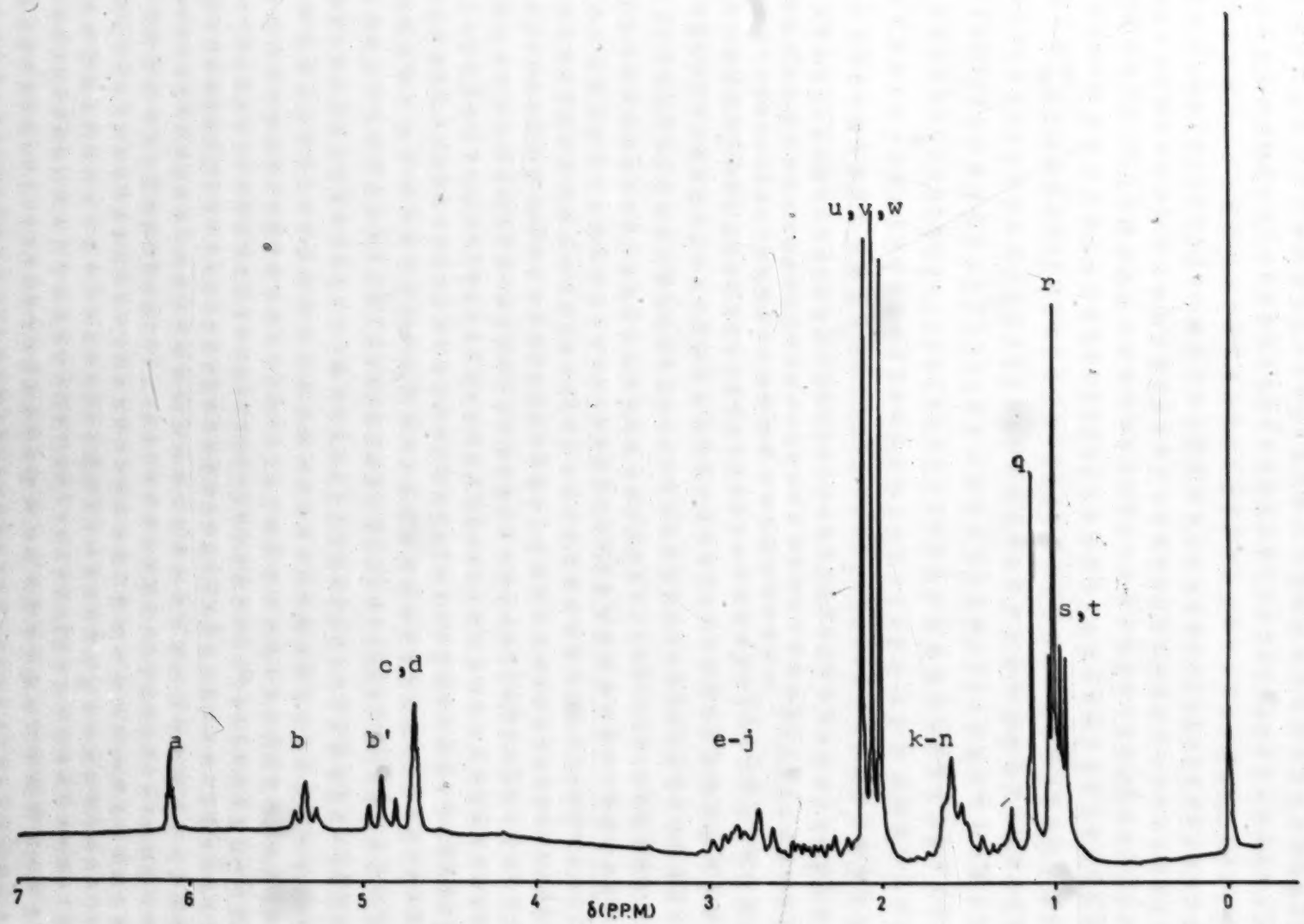

u,v,w
r
q
s,t
c,d
a
b
b'
e-j
k-n
7
6
5
4
δ(PPM)
3
2
1
0

Table III. Nmr data of 0,0,0-triacetylcyafrin A in $CDCl_3$

Signal	Chemical shift (δ)	Number of protons	Multiplicity	Coupling constant (Hz)	Coupled with
a	6.12	1	t	1.5	c,d
b	5.33	1	t	6.5	e-j region
b'	4.89	1	t	8	e-j region
c,d	4.70	2	d	1.5	a
e-j	3.0-2.1	6	u	?	b,b',s,t,?
k-n	1.9-1.1	4	u	?	?
q	1.14	3	s		
r	1.02	3	s		
s	1.01	3	d	7	e-j region
t	0.97	3	d	7	e-j region
u	2.12	3	s		
v	2.07	3	s		
w	2.02	3	s		

the same basic structure; of two protons absorbing in the methylene proton region for 0,0-diacetylcyathin A_3, one has been replaced by a hydroxyl group (an acetoxyl group in 0,0,0-triacetylcyafrin A_4), the other by a proton which absorbs in the same region as other protons geminal to an acetoxyl group.

If the first conclusion is correct some tentative structure assignments for cyafrin A_4 may be arrived at. Looking at the structure of 0,0-diacetylcyathin A_3 and at the assignment of proton signals in the molecule (see structure 13) it is found that the molecule contains six methylene carbons that could in principle bear the extra acetoxyl group observed in 0,0,0-triacetylcyafrin A_4. They are carbons 1, 2, 7, 8, 10 and 15. Of these, C-10 can be ruled out: both protons on C-10 (signal g,j) are still present in the nmr spectrum of 0,0,0-triacetylcyafrin A_4, as is evidenced by the multiplicity of signal b (fig. 8, table III). C-15 is not a possible site as the nmr signal c,d (fig. 8) of 0,0,0-triacetylcyafrin A_4 contains two protons. It seems then that cyafrin A_4 could have the same skeleton as cyathin A_3, but would bear an extra oxygen on one of carbons 1, 2, 7 and 8.

To find further support for the tentative structure assignment and to obtain further information about the position of the extra oxygen function we decided to prepare

a second derivative of cyafrin A_4. An attractive possibility presented itself at once. It has been found that cyathin A readily forms an 0,0-isopropylidene derivative [12, p.102] [3], which results in the formation of an extra six-membered ring. If cyafrin A_4 does possess a similar basic skeleton it should form this derivative without difficulty, as the extra hydroxyl group is not likely (see above) to be in a position to interfere with the formation of an isopropylidene derivative. Another reason for preparing this derivative was this: a chemical conversion of cyafrin A_4 to one of the known cyathins would prove our tentative structure assignment for cyafrin A_4. Selective protection of two of the hydroxyl groups of cyafrin A_4 as the 0,0-isopropylidene derivative would leave the third hydroxyl free for chemical transformations.

The 0,0-isopropylidene derivative (cyafrin A_4 acetonide) was prepared by dissolving cyafrin A_4 in 2,2-dimethoxypropane. The reaction was catalyzed with p-toluenesulphonic acid. Direct application of the reaction mixture to a prep. tlc plate and elution (solvent system A) resulted in the isolation of pure (by tlc) cyafrin A_4 acetonide.

The mass spectrum of cyafrin A_4 acetonide (see fig. 9) shows a strong parent peak at m/e 374 (hrms: $C_{23}H_{34}O_4$) as expected for a mono-0,0-isopropylidene deriva-

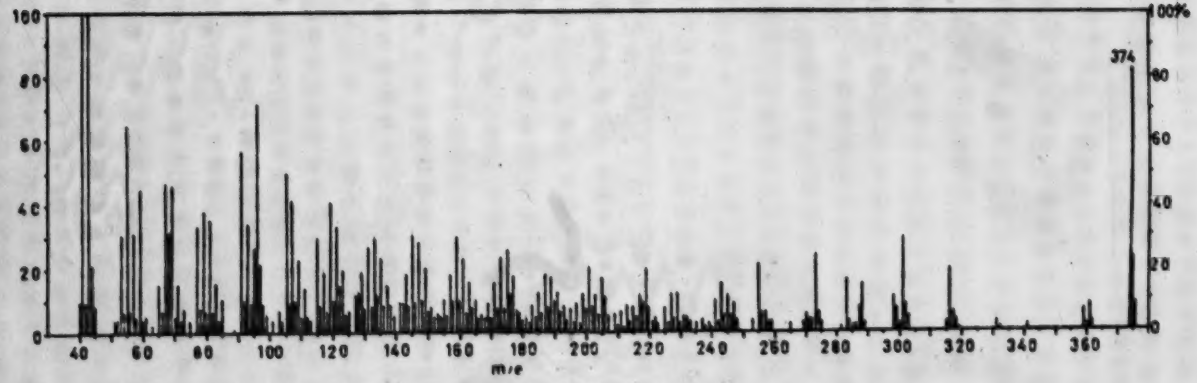


Figure 9. Mass spectrum of 0,0-isopropylidenecyafrin A

tive of a $C_{20}H_{30}O_4$ compound, and no evidence of impurities.

The nmr spectrum of cyafrin A_4 acetonide (see fig. 10) confirms that it is an 0,0-isopropylidene derivative: the two three proton singlets around δ 1.45 are attributed to the newly introduced methyl groups.

As was the case with the spectrum of the triacetyl derivative of cyafrin A_4 the nmr spectrum of cyafrin A_4 acetonide can be analyzed by comparison with that of cyathin A_4 acetonide. This compound was first prepared by Mercer,[3] and table V is from data collected by him[12, p.60]. Comparison of the data in this table with the nmr data for cyafrin A_4 acetonide (table IV) shows many similarities. The data in the last two columns of table IV were obtained by means of spin-spin decoupling (double irradiation) experiments. The differences between the data in these two tables are very instructive. The spectrum of cyafrin A_4 acetonide shows the extra signal b' at δ 3.9, and there appear to be two protons less in the δ 1.2-1.8 region. This confirms our previous conclusions: signal b' is attributable to a proton on a carbon that also bears a hydroxyl group. Signal b' is coupled with two protons (δ 2.3-2.5) by equal or almost equal spin-spin coupling constants.

The question remained: which of the carbons 1, 2, 7 and 8 is the one bearing the hydroxyl group? The chemi-

Figure 10. Nmr spectrum of 0,0-isopropylidenecyafrin A ($CDCl_3$)

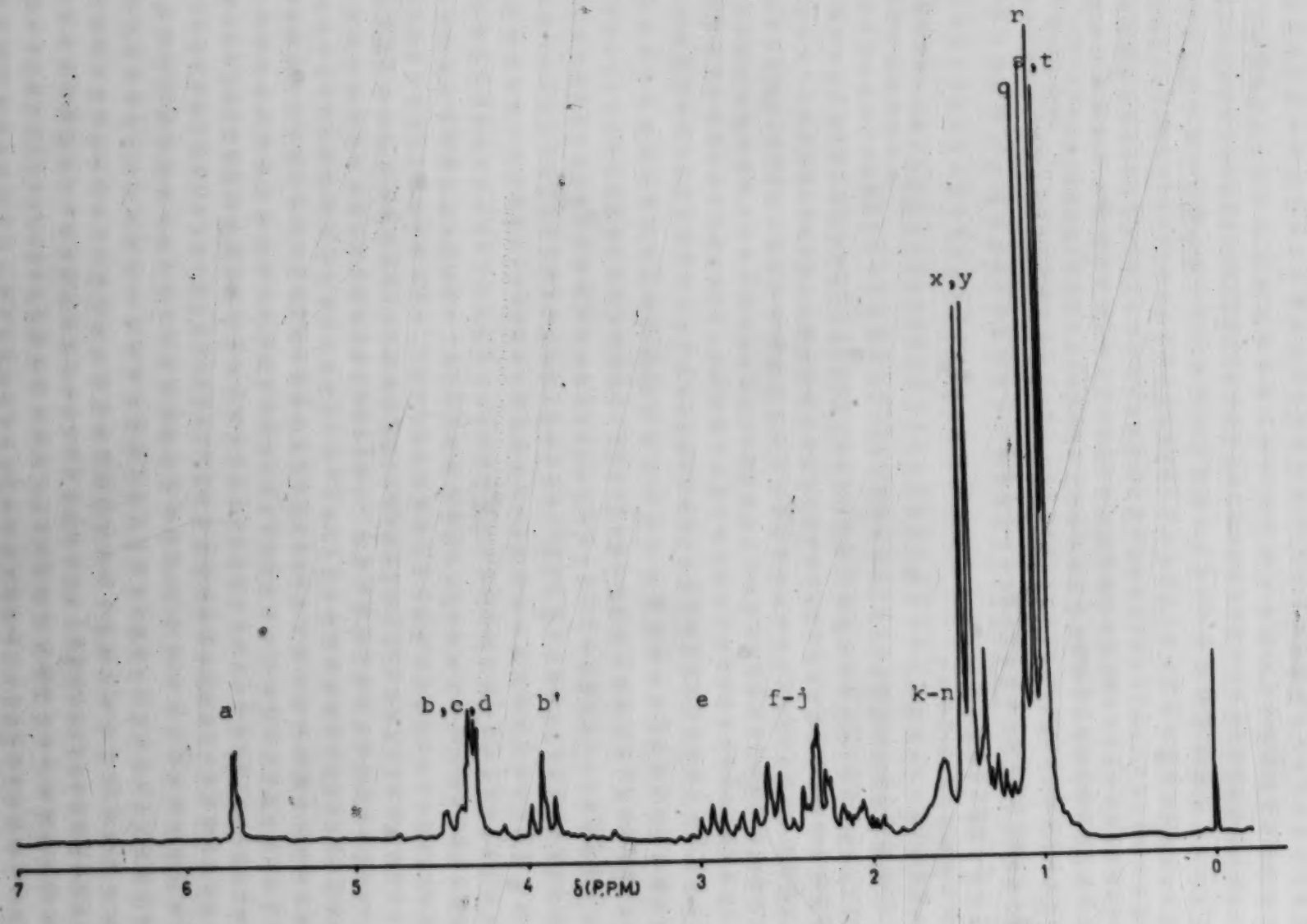

r
s,t
q
x,y
a
b,c,d
b'
e
f-j
k-n
7
6
5
4
δ(P.P.M)
3
2
1
0

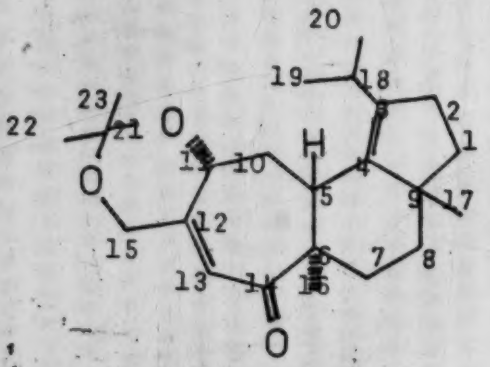
Table IV. Nmr data of O,O-isopropylidenecyafrin A in $CDCl_3$

Signal	Chemical shift (δ)	Number of protons	Multiplicity	Coupling constant (Hz)	Coupled with
a	5.71	1	u	small	b,c,d region
b,c,d	4.3-4.5	3	u	?	a,?
b'	3.90	1	t	7.5	2.3-2.5 region
e	2.92	1	q,q	6.5	s,t
f-j	2.8-1.8	5	u	?	b',?
k-n	1.8-1.2	4	u	?	?
q	1.09	3	s		
r	1.00	3	s		
s,t	1.02	6	d	6.5	e
x	1.46	3	s		
y	1.42	3	s		

Table V. Nmr data of 0,0-isopropylidenecyathin A$_3$ in $CDCl_3$

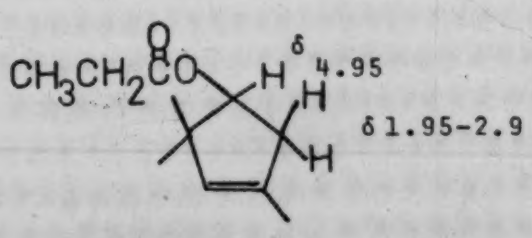
Signal	Chemical shift (δ)	Number of protons	Multiplicity	Coupling constant (Hz)	Assignment[a]
a	5.68	1	u	small	13
b,c,d	4.25-4.5	3	u	?	11,15
e	2.92	1	q,q	6.5	18
f-j	2.8-1.8	5	u	?	2,5,10
k-p	1.8-1.1	6	u	?	1,7,8
q	1.10	3	s		(16, 17)
r	1.06	3	s		
s	1.02	3	d	6.5	(19, 20)
t	0.99	3	d	6.5	
x	1.47	3	s		(22, 23)
y	1.40	3	s		

[a] Numbers refer to numbered positions in structure 14 (0,0-isopropylidenecyathin A$_3$).



14

cal shifts of the methylene protons which are coupled with the proton responsible for signal b', as we have found them in the spectra of the triacetyl- and the acetonide derivatives (2.1-3.0 and 2.3-2.5 ppm respectively, see tables III and IV) make positions 7 and 8 unlikely to be the ones: the presence of a hydroxyl group on an adjacent carbon would not be expected to deshield these protons appreciably from, e.g., the shift of 1.44 ppm found for the methylene protons in cyclohexane [18, p.214]. The literature was searched for nmr data on suitable model compounds to make a decision between positions 1 and 2 possible. No data on 2-cyclopenten-1-ol or similar compounds were found, but data on some 3-cyclopenten-1-ol systems are available. The relevant chemical shifts are indicated on the corresponding structures 15 (from ref. 19) and 16 (from ref. 20). These values correspond closely to our values: signal b' is found at δ 4.89 in triacetylcyafrin A_4 and at δ 3.90 in cyafrin A_4 acetonide. The methylene protons resonate at δ 2.1-3.0 and δ 2.3-2.5



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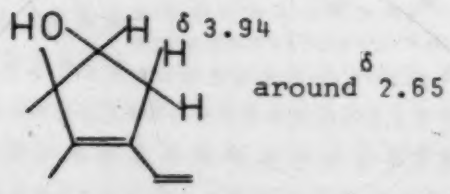
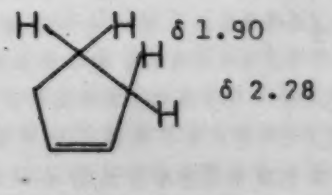


16

in these two derivatives respectively. It is not likely that the protons in 2-cyclopenten-1-ol systems would have similar chemical shifts, as is shown by the large difference between the two sets of methylene protons in cyclopentene itself (structure 17, from ref. 21).



17

It was therefore tentatively assumed that the third hydroxyl group in cyafrin A[4] is positioned on C-1.

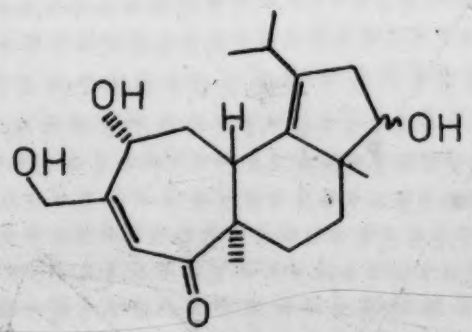
Oxidation of the free hydroxyl group in 0,0-isopropylidenecyafrin A_4 offered a second method of ascertaining the position of the third hydroxyl group of cyafrin A_4. Oxidation of 0,0-isopropylidenecyafrin A_4 was achieved using "Jones" conditions (see detailed experimental) using chromic acid. After filtration and separation of the product mixture by prep. tlc (solvent system A) a small quantity of chromatographically pure material was isolated. Mass spectral analysis of this material indicated that it was the expected oxidation product: a parent peak was found at m/e 372 (which corresponds to the molecular weight of the starting material minus two protons).

The infrared spectrum of the oxidation product showed carbonyl absorption at 1735 cm^{-1}. This is consistent with the presence of a non-conjugated carbonyl group in a five-membered ring.

The nmr spectrum of the oxidation product showed as new feature a two proton singlet at δ 3.00, while the triplet found in the spectrum of cyafrin A_4 acetonide at δ 3.90 had disappeared. The two proton singlet may be assigned to the two protons adjacent to the newly introduced carbonyl group. The fact that these are magnetically equivalent is an indication that they may be positioned on a five-membered ring. In addition, the chemical shift of these

two protons is such (δ 3.00) that it can only be accommodated in the cyathin skeleton by assuming them to be positioned on C-2, which is flanked both by the new carbonyl group and by the C=C double bond. This confirms that the extra hydroxyl group in cyafrin A_4 is positioned on C-1.

We can now arrive at a more precise formulation (structure 18) of cyafrin A_4.



18

The only problem that remains to be solved is that of the stereochemistry of the C-1 hydroxyl group, i.e., is the hydroxyl group orientated cis to the methyl group on C-9 or trans to it?

It is known that when nmr spectra are recorded in pyridine the chemical shifts of protons which are

positioned close to carbonyl or hydroxyl groups may change considerably when compared to their shifts in non-aromatic solvents such as chloroform. It is assumed that the pyridine complexes with these functional groups, thus changing the magnetic environment of nearby protons. In the case where a methyl group is situated on a carbon vicinal to a carbon bearing a hydroxyl group, a definite relationship has been found to exist[22] between the dihedral angle (formed by the C-OH bond and the C-CH_3 bond) and the difference in chemical shift of the methyl protons[3] when measured first in chloroform and then in pyridine solution. Thus for a synclinal relationship between hydroxyl and methyl groups, a downfield shift of 0.20 PPM or larger may be expected. For an antiperiplanar relationship the shift will be 0.10 - 0.12 PPM or less. By recording the nmr spectrum of 0,0-isopropylidenecyafrin A[4] in pyridine-d_5 and noting how the chemical shifts of the methyl groups change compared to the shifts in $CDCl_3$ (as given in table IV) it was hoped that the orientation[3] of the hydroxyl group at C-1 could be deduced. Table VI shows how the shifts of the methyl groups compare for the chloroform and pyridine spectra. The doublet signals are due to the protons on the isopropyl group. Two interpretations are possible for the tertiary methyl signals. Either both signals have shifted by 0.21 PPM (from δ 1.00 to δ 1.21 and from δ 1.09 to δ 1.30 respectively) or one has shifted by 0.30 PPM (from δ 1.00 to δ 1.30) and the

Table VI. Chemical shifts of methyl protons in 0,0-isopropylidenecyafrin A[4]

Shift in $CDCl_3$ (δ)	Multiplicity	Shift in C_5D_5N (δ)	Multiplicity
1.09	s	1.30	s
1.02	d[a]	1.21	s
1.00	s	1.01	d
		0.91	d

[a] Integrates to six protons.

other by 0.12 PPM (from δ 1.09 to δ 1.21). The first of these two interpretations is the more attractive since it eliminates the need to establish which signal corresponds to the C-17 methyl group and which to the C-16 methyl group. It is not unusual that the C-16 methyl group shifts as well as the C-17 methyl group, since it is near the carbonyl group. This would then lead to the conclusion that the hydroxyl group at C-1 must be orientated cis to the vicinal methyl group.

The second possible interpretation must not be neglected. Assuming that one methyl signal had shifted by 0.12 PPM and the other by 0.30 PPM it becomes necessary to establish which signal belongs to which methyl group. To determine this the spectrum of 0,0-isopropylidenecyathin A[3]

(prepared by the method of Mercer[12, p.102]) was recorded in pyridine and compared with that recorded in chloroform (see table V). Cyathin A_3 is not functionalized in the five-membered ring, so the shift of the C-17 methyl protons is not expected to be influenced appreciably by the change of solvent. If we see a shift of one of the methyl signals then that shift must be associated with the C-16 methyl group. The nmr spectral results are given in table VII. The isopropylidene signals are readily recognized by their multiplicity.

Table VII. Chemical shifts of methyl protons in 0,0-isopropylidenecyathin A_3

Shift in $CDCl_3$ (δ)	Multiplicity	Shift in C_5D_5N (δ)	Multiplicity
1.10	s	1.18	s
1.06	s	1.06	s
1.02	d	0.96	d
0.99	d	0.92	d

The chemical shift of the C-17 methyl protons should not change markedly and must be that of the signal at δ 1.06. The signal of the C-16 methyl protons has shifted from δ 1.10 to δ 1.18, i.e., 0.08 PPM downfield. Thus in cyafrin A_4 acetonide the signal which shifts by 0.12 PPM must be the signal due to the C-17 methyl protons, and that which

shifts 0.30 PPM must be from the C-16 methyl protons. Hence the hydroxyl group at C-1 must be orientated *cis* to the C-17 methyl group.

It is possible to confirm this stereochemical assignment using ^{13}C-nmr. Recently Djerassi *et al*[23] measured ^{13}C-nmr spectra of a number of steroids in which a hydroxyl group and a methyl group were positioned on adjacent carbons in a five-membered ring. They noted that if the relationship between hydroxyl and methyl group was antiperiplanar, the chemical shift of the methyl carbon was only 0.4 PPM upfield from the case where the hydroxyl group was absent. If the relationship between them was synclinal, the methyl group was shifted upfield about 6 PPM.

The ^{13}C-nmr spectra of several known cyathins, including 0,0-diacetylcyathin A[3], have recently been measured in these laboratories, and the signals assigned to specific carbon atoms[24].

A large quantity (45 mg) of 0,0,0-triacetylcyafrin A[4] was prepared and a ^{13}C-nmr spectrum was obtained on this material. The shifts were compared with those of 0,0-diacetylcyathin A[3] (see table VIII). Many signals have similar shifts to those of 0,0-diacetylcyathin A[3]. These have tentatively been assigned to the corresponding carbons.

Table VIII. ^{13}C-nmr data of 0,0-diacetylcyathin A$_3$ and 0,0,0-triacetylcyafrin A$_4$ in $CDCl_3$.

0,0-diacetylcyathin A$_3$		0,0,0-triacetylcyafrin A$_4$	
Chemical shift (PPM)	Assignment[a]	Chemical shift (PPM)[b]	Chemical shift (PPM)[c]
208.7	14		80.8
170.1	(21,		34.4
169.8	22)		21.9
143.4	12	143.5	17.1
140.7	(3,	138.0	
135.5	4)	134.4	
127.4	13	127.3	
72.0	11	71.8	
64.7	15	64.7	
54.3	6	53.6	
49.3	9	50.4	
38.7	5	39.6	
38.0	(1,		
36.2	7)	35.4	
34.7	8	34.9	
31.7	10	31.7	
28.2	2		
27.2	18	27.1	
24.0	17		
21.7	(19,	21.1	
21.7	20)	21.1	
20.8	(23,	20.8	
20.8	24)	20.8	
14.8	16	15.0	

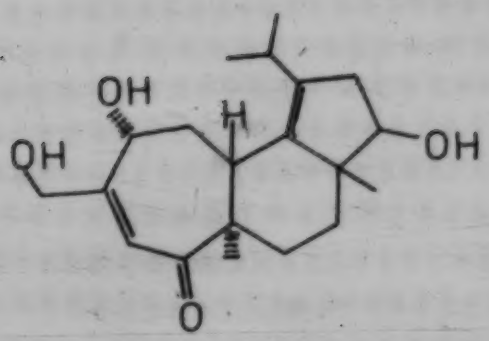
[a] Numbers refer to carbons numbered as in structure 13, p.26.

[b] Only the shifts of signals closely corresponding to signals in the first column are listed here.

[c] The shifts of signals not listed in the previous column are listed here.

Several major differences are also apparent. First of all, only 22 signals were found for O,O,O-triacetylcyafrin A_4, although the compound contains 26 carbons. At the time the spectrum was recorded samples larger than 45 mg were usually required for good results. The missing signals are those of the carbonyl carbons (C-14 and the acetyl carbons), which are relatively weak signals. The signal assigned to C-1 in O,O-diacetylcyathin A_3 has apparently shifted to δ 80.8, close to the signal for C-11, the other methine carbon bearing an acetoxyl group in O,O,O-triacetylcyafrin A_4. The signal for C-2 in O,O-diacetylcyathin A_3 has shifted from δ 28.2 to δ 34.3 in O,O,O-triacetylcyafrin A_4, due to the influence of the acetoxyl group at C-1. In the ^{13}C-nmr of O,O,O-triacetylcyafrin A_4 a new signal is found in the methyl region at δ 21.9: this must be the methyl carbon of the acetoxyl group. Finally the signal assigned to C-17 in in O,O-diacetylcyathin A_3 has shifted upfield from δ 24.0 to δ 17.1, *i.e.*, by 6.9 PPM, in O,O,O-triacetylcyafrin A_4, which compares with a shift of about 6 PPM in the case of a synclinal relationship observed by Djerassi[23], and which is a typical γ-gauche effect.

This confirms our earlier finding that the hydroxyl group at C-1 in cyafrin A_4 must be orientated *cis* to C-17. Structure **19** is therefore proposed for cyafrin A_4.



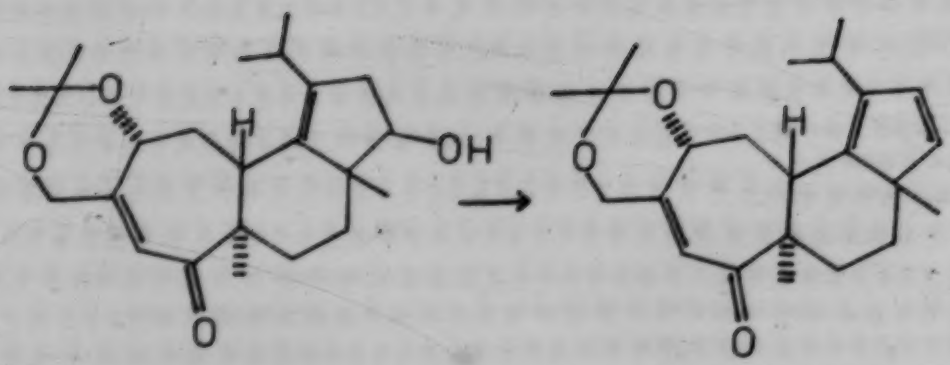
19

Correlation of cyafrin A_4 with allocyathin B_3

Abundant spectral evidence has been presented to support structure 19 as that of cyafrin A_4. The finding of cyathin A_3 and allocyathin B_3 as major metabolites of C. africanus shows that the structure for cyafrin A_4 is biogenetically reasonable. In order to prove conclusively that cyafrin A_4 has structure 19, a chemical correlation of cyafrin A_4 with one of the known cyathins was desirable.

We proposed to do this (as illustrated in scheme I) by dehydration of 0,0-isopropylidenecyafrin A_4 to give 0,0-isopropylideneallocyathin B_3, a compound which has been prepared from allocyathin B_3 and described by Mercer[12].

Many reagents are known which can effect dehydration. However, many of them employ acidic conditions



Scheme I. Proposed conversion of 0,0-isopropylidenecyafrin A[4] to 0,0-isopropylideneallocyathin B[3].

which should be avoided in this case because the 0,0-isopropylidene protecting group is acid labile. A procedure that employs basic conditions developed by Von Rudloff[25] has been used successfully for the dehydration of various natural products and derivatives[26]. This method consists of heating the alcohol in the presence of alumina impregnated with 1 to 2% of pyridine. We decided to replace pyridine by collidine to minimize loss of the reagent by evaporation. A small amount of cyafrin A[4] acetonide was heated with this catalyst at 160°C for 30 minutes (these conditions are much milder than the ones suggested by Von Rudloff). The products were washed from the catalyst with methylene chloride and then methanol. The product mixture was examined by tlc, using authentic allocyathin B[3] acetonide as reference substance. However, no trace of the desired product nor any starting material could be detected in the product. Dis-

couraged by these findings we decided to look for less vigorous dehydration methods.

Another well known dehydrating agent is thionyl chloride, which may be used in combination with a base such as pyridine. When we first attempted this reagent, pyridine was used as solvent and a large excess of thionyl chloride was added. The reaction was allowed to proceed at 0 °C for three hours. After this time part of the reaction mixture was worked up and examined by tlc. It appeared that some of the starting material had disappeared, but little else could be detected. After a further four hours reaction at room temperature the product was again examined. The result this time was that most of the starting material had disappeared. The bulk of the reaction mixture now appeared to have an R_f of 0.0 (solvent system A) but a very faint spot was just discernable at the R_f expected of allocyathin B_3 acetonide (R_f 0.8).

Dehydration was then attempted using methylene chloride as solvent, with excess pyridine and thionyl chloride for 24 hours at room temperature. Examination of an aliquot of the reaction mixture showed very little change after 6 hours. After 24 hours tlc indicated a similar result as before: very polar material and a faint spot around R_f 0.8.

It was decided to use acetone as solvent (as this should insure the retention of the acetonide protecting group) and smaller amounts of reagents. The reaction was monitored by direct application of aliquots of the reaction mixture to tlc plates. This method proved useful: using 10 equivalents of freshly distilled thionyl chloride and 30 equivalents of pyridine in dry acetone at 0 ^{o}C and monitoring the reaction by tlc (solvent system A) it was found that after 50 minutes the reaction was complete. The tlc of the reaction product showed not only highly polar material, but also a substantial amount of material with R_f 0.8, which is the R_f value of allocyathin B_3 acetonide. The material at R_f 0.8 was isolated by prep. tlc. The yield was somewhat less than 2 mg from 3.5 mg of starting material.

The mass spectrum of the dehydration product (see fig. 11) appeared promising. The apparent parent peak at m/e 356 is that expected for allocyathin B_3 acetonide. In fact, this spectrum was identical with the mass spectrum of allocyathin B_3 acetonide recorded by Mercer.

The nmr spectrum of the "dehydration product" (see fig. 12) was, however, disappointing. This spectrum (recorded at 60 MHz on a small quantity of material) showed all the features expected of a cyathin acetonide: two methyl signals around δ 1.45, two more just above δ 1.00 and ab-

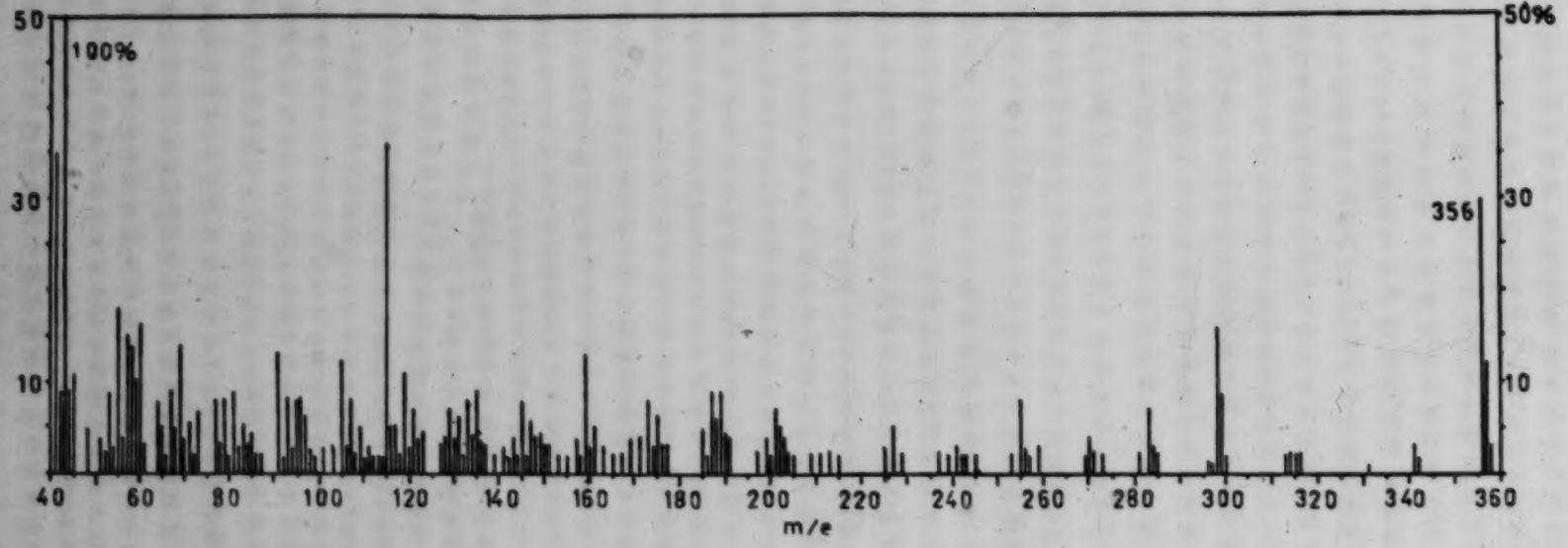


Figure 11. Mass spectrum of the dehydration product of cyafrin A_4 acetonide

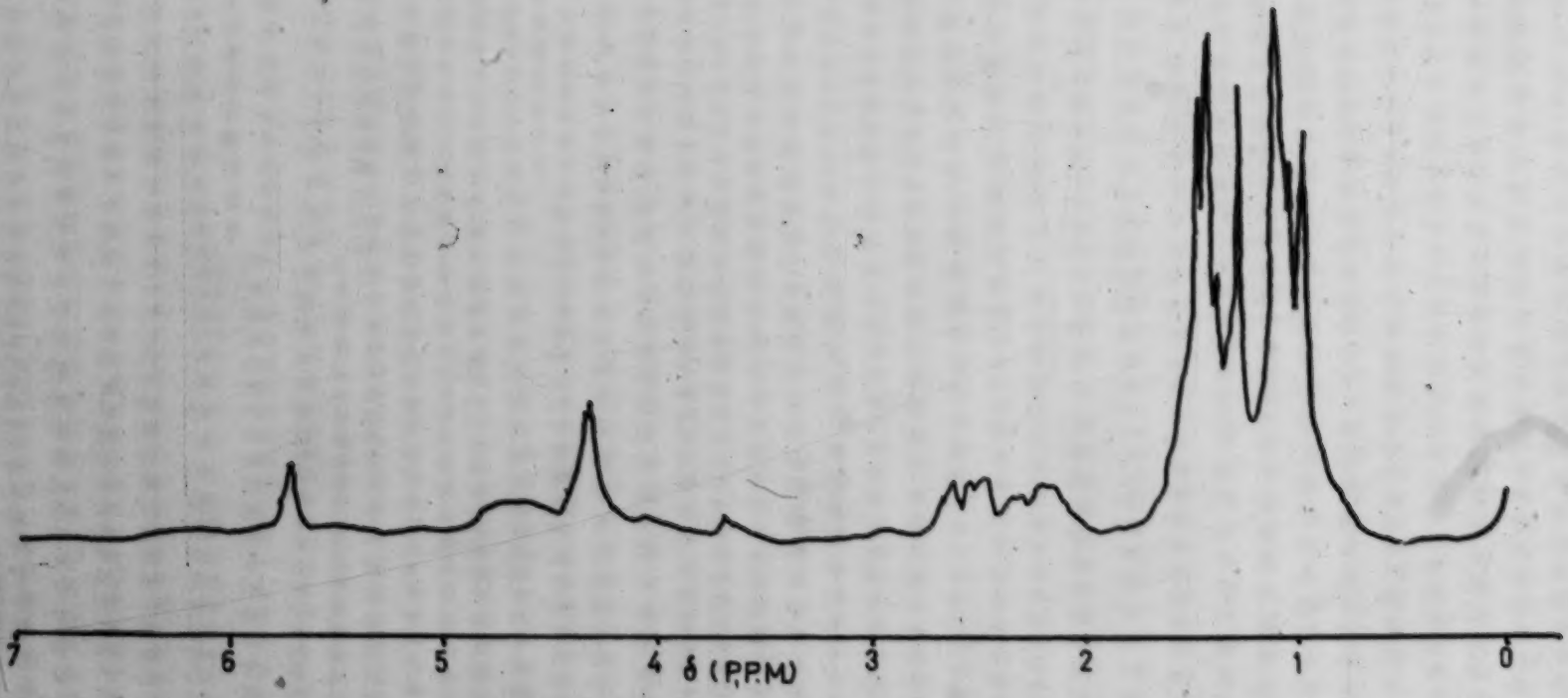
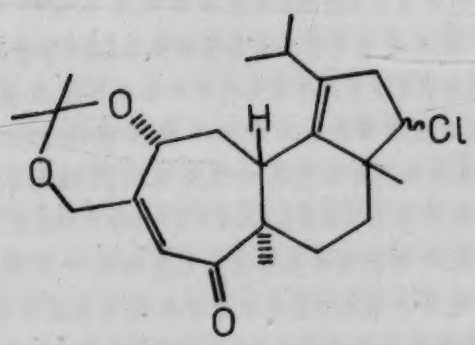


Figure 12. Nmr spectrum of the "dehydration product" of cyafrin A$_4$ acetonide (CDCl$_3$)

sorption around δ 1.0 that indicate the presence of an isopropyl group. Also, fairly sharp signals are found at δ 4.3 and 5.7 (compare signals b,c,d and a in tables IV and V). However, allocyathin B_3 acetonide has two olefinic protons on C-1 and C-2 that are known to resonate around δ 6.3, and the reaction product (fig. 12) does not show signals in this region. In addition, fig. 13 shows a broad signal around δ 4.8 which is not present in the spectrum of allocyathin B_3 acetonide.

The uv spectrum of the material in hexane confirmed that the material is not identical with allocyathin B_3 acetonide; it showed absorption maxima at 231 nm (ε ~5000) and 330 nm (ε ~100). These are the normal values for the α,β-unsaturated ketone found in all cyathins. No trace was found of a shoulder at 256 nm, as would be expected for the conjugated diene present in allocyathin B_3 acetonide[7,9].

It is known than when thionyl chloride is used as dehydrating agent, sometimes the reaction that takes place is substitution instead of elimination. That is, in our case, we could have obtained 1-chlorocyathin A_3 acetonide (see structure 20) instead of allocyathin B_3 acetonide. This would account for the evidence described: 1-chlorocyathin A_3 acetonide would not show diene absorption in the ultraviolet region, nor would it show signals at δ 6.3 in the nmr spectrum, but it could well show a signal around δ 4.8, due to



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the proton remaining on C-1. The mass spectrum may be explained by assuming that 1-chlorocyathin A_3 acetonide, when heated under vacuum in the mass spectrometer or on electron impact, undergoes elimination of HCl to produce allocyathin B_3 acetonide or the corresponding radical ion.

At this point we recalled that the mass spectrum of 0,0,0-triacetylcyafrin A_4 (see fig. 7) showed the highest mass peak at m/e 400 instead of 460 as expected, which we attributed to the molecule losing a molecule of acetic acid in the mass spectrometer. If this molecule of acetic acid originated from the acetoxyl group on C-1 and a proton on C-2, this would result in the formation of 0,0-diacetylallocyathin B_3. Comparison of fig. 7 with the mass spectrum of allocyathin B_3 diacetate as recorded by Taube[7] showed them to be identical.

The mass spectral correlation strongly suggests that cyafrin A_4 does have the same basic skeleton as allocyathin B_3. However, the direct correlation has not been achieved, and the possibility that there are stereochemical differences (relative or absolute) has not been eliminated.

Further attempts to obtain more of what is assumed to be 1-chlorocyathin A_3 acetonide were not undertaken because of a lack of starting material: during the later stages of this work the amount of cyafrin A_4 isolated from growths of C. africanus had decreased. The area on prep. tlc that earlier contained pure cyafrin A_4 had now begun to contain increasing amounts of other compounds, which proved very difficult to remove. Presumably mutations had occurred in the fungus and it will be necessary to obtain a new stock culture before the direct correlation can be completed.

Isolation and proposed structure of cyafrin B_4

We now turned our attention to component C found on tlc at R_f 0.4 (solvent system A, triple elution).

Mass spectral analysis of component C, which was isolated by prep. tlc (solvent system A, triple elution), in most cases showed several apparent parent ions. The major one by far was at m/e 332 (hrms: $C_{20}H_{28}O_4$). Other high mass

peaks appeared at m/e 348, 334 and 314 (hrms: $C_{20}H_{28}O_5$, $C_{20}H_{30}O_4$ and $C_{20}H_{26}O_3$ respectively), with further prominent peaks at m/e 219 ($C_{15}H_{23}O$) and 205 ($C_{14}H_{21}O$). This material thus appeared to be composed mainly of a compound with molecular formula $C_{20}H_{28}O_4$, which according to our system for naming cyafrins was called cyafrin B_4. It was, however, contaminated with small amounts of several other compounds.

Cyafrin B_4 was purified by prep. tlc, using solvent system C, quadruple elution. After this purification the mass spectrum looked very much cleaner (fig, 13). The fragments m/e 205, $C_{14}H_{21}O$ and m/e 219, $C_{15}H_{23}O$ obviously do originate from cyafrin B_4.

The ir spectra of cyafrin B_4, recorded in $CHCl_3$ solution and neat (fig. 14 and 15 respectively) proved interesting (the latter spectrum was obtained by evaporating the solvent from a solution of cyafrin B_4 which had been applied to the surface of an ir cell). The solution spectrum shows free hydroxyl (3600 cm^{-1}), hydrogen bonded hydroxyl (3400 cm^{-1}) and strong carbonyl (1685 cm^{-1}) absorptions. As was the case with cyafrin A_4 the free hydroxyl absorption disappears in the neat spectrum. The carbonyl absorption is also less intense. This phenomenon is often observed for cyathin-like compounds.

The uv spectrum of cyafrin B_4 shows the ge-

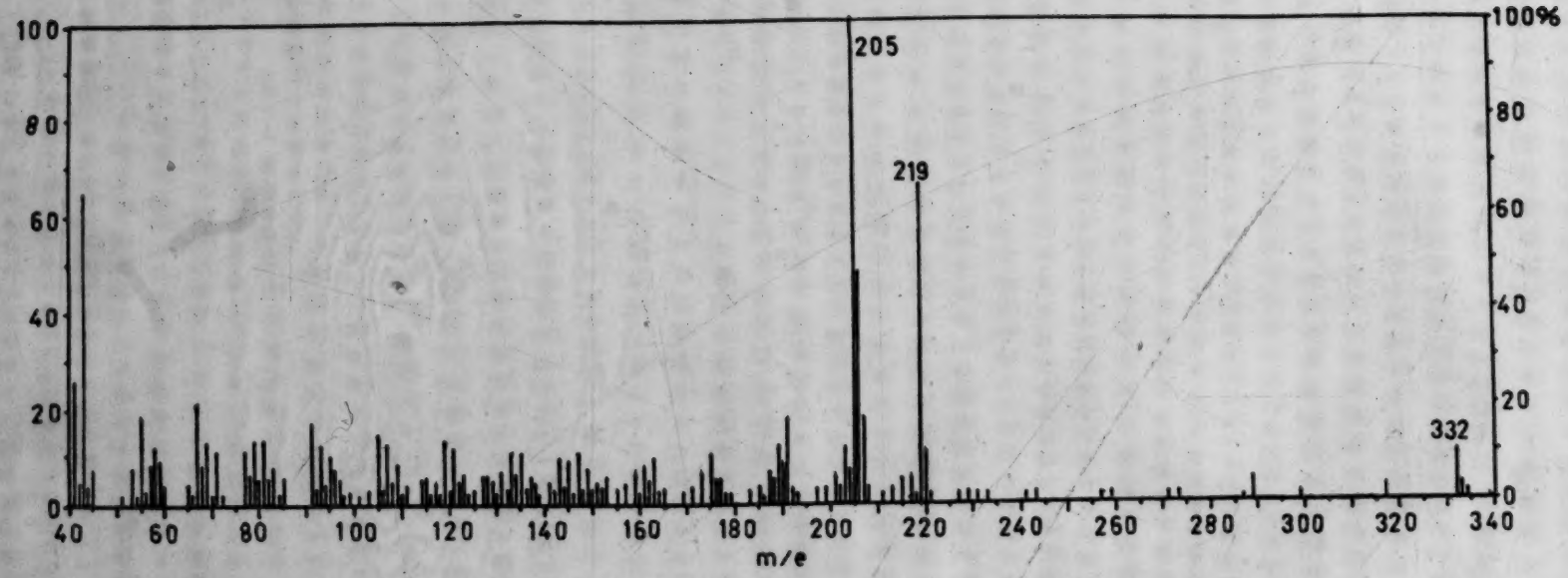


Figure 13. Mass spectrum of cyafrin B_4.

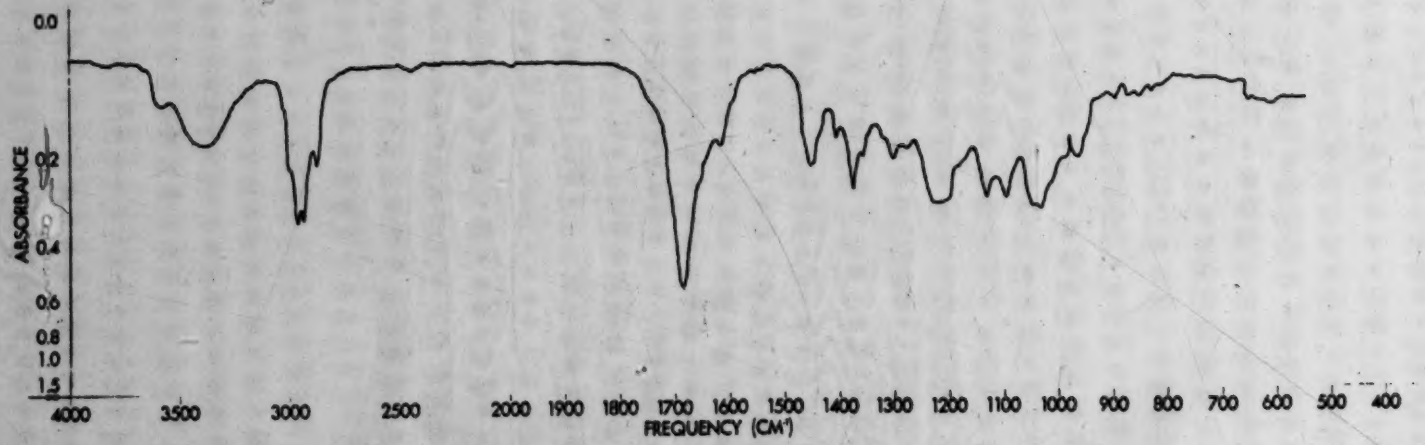


Figure 14. Ir spectrum of cyafrin B_4 ($CHCl_3$ solution)

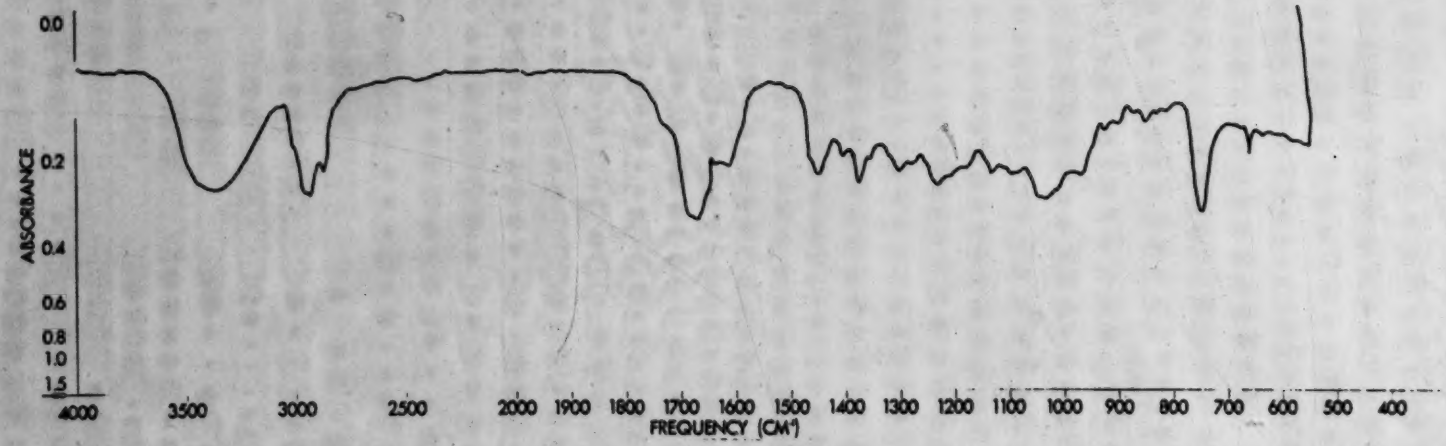


Figure 15. Ir spectrum of cyafrin B_4 (neat)

neral features of all cyathin-like compounds: a maximum at 231 nm (ε ~7000) and a shoulder around 305 nm (ε ~100). Both may be attributed to the α,β-unsaturated ketone function present in the cyathins.

In view of the small amount of material present, an nmr spectrum of cyafrin B_4 was not recorded since it would not be expected to yield much information. Instead an acetylated derivative of cyafrin B_4 was prepared, since this would give information about the number of hydroxyl groups in the molecule and enable us to record well resolved spectra. Cyafrin B_4 was acetylated in the manner described previously for cyafrin A_4. The product was isolated by prep. tlc (solvent system A) and further purified by the same method (solvent system B, double elution).

The mass spectrum of the acetylated product (see fig. 16) has a parent peak at m/e 416 ($C_{24}H_{32}O_6$). This indicates that the product is a diacetyl derivative of cyafrin B_4. The peak at m/e 356 is attributed to loss of one molecule of acetic acid from the parent ion. The peaks at m/e 205 and 219 are still present, indicating that a major portion of the molecule has not been affected by the acetylation.

The infrared spectrum of 0,0-diacetylcyafrin B_4 (see fig. 17) confirms the presence of the acetoxyl

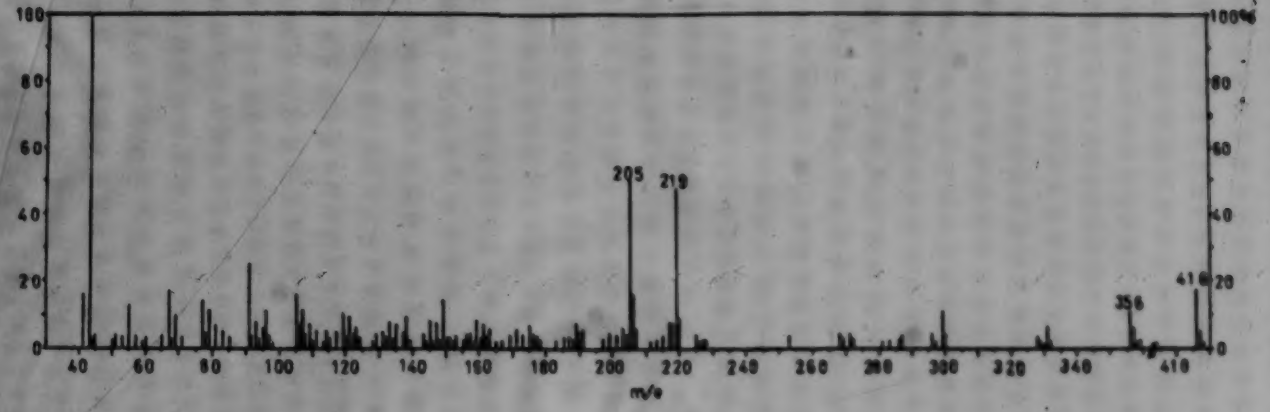


Figure 16. Mass spectrum of O,O-diacetylcyafrin B

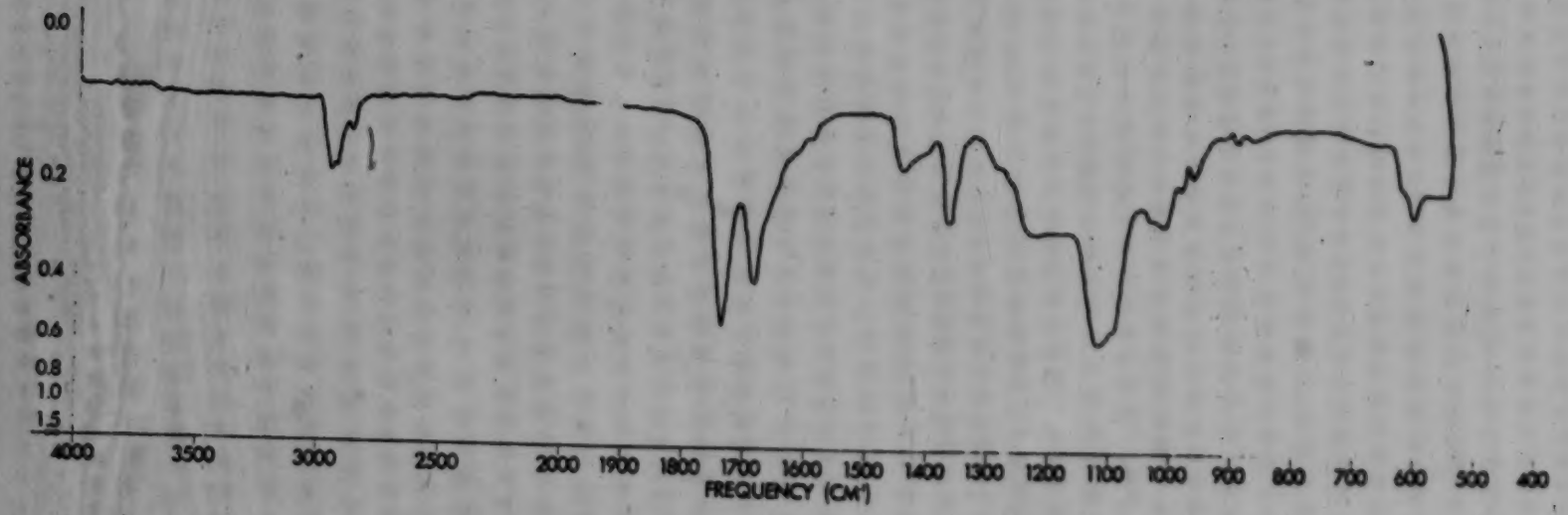


Figure 17. Ir spectrum of 0,0-diacetylcyafrin B

groups (carbonyl absorption at 1740 cm^{-1}). The absorption at 1685 cm^{-1} is attributed to the α,β-unsaturated ketone function common to all cyathins. The absence of absorption around 3500 cm^{-1} shows that all hydroxyl groups in the molecule have been acetylated.

The nmr spectrum of 0,0-diacetylcyafrin B_4 (see fig. 18) has some interesting features. First, it confirms the presence of two acetoxyl groups by the appeareance of two three-proton singlets at δ 2.06 and 2.12. Second, the overall appearance of the spectrum is very similar to that of other acetyl derivatives of cyathins. Comparison of the data for this spectrum (table IX) with, for example, table I is very instructive. The signals a, b, c, d, u and v appear in the spectrum of 0,0-diacetylcyafrin B_4 (table IX) virtually unchanged from those observed in the spectrum of 0,0-diacetylcyathin A_3 (table I). The region between δ 2.25 and 3.0 of the nmr of 0,0-diacetylcyafrin B_4 contains signals for no more than three protons, compared to six in 0,0-diacetylcyathin A_3 (remembering that the molecular formula for cyafrin B_4 indicates two hydrogens less than for cyathin A_3). The "missing" signal in the nmr of 0,0-diacetylcyafrin B_4 has perhaps shifted to become signal d' at δ 3.13. The sharp signal j' at δ 2.21 is difficult to account for at this stage: possibly it could originate from an impurity in the sample. Signals corresponding to the signals q, r, s and t

Figure 18. Nmr spectrum of 0,0-diacetylcyafrin B
4

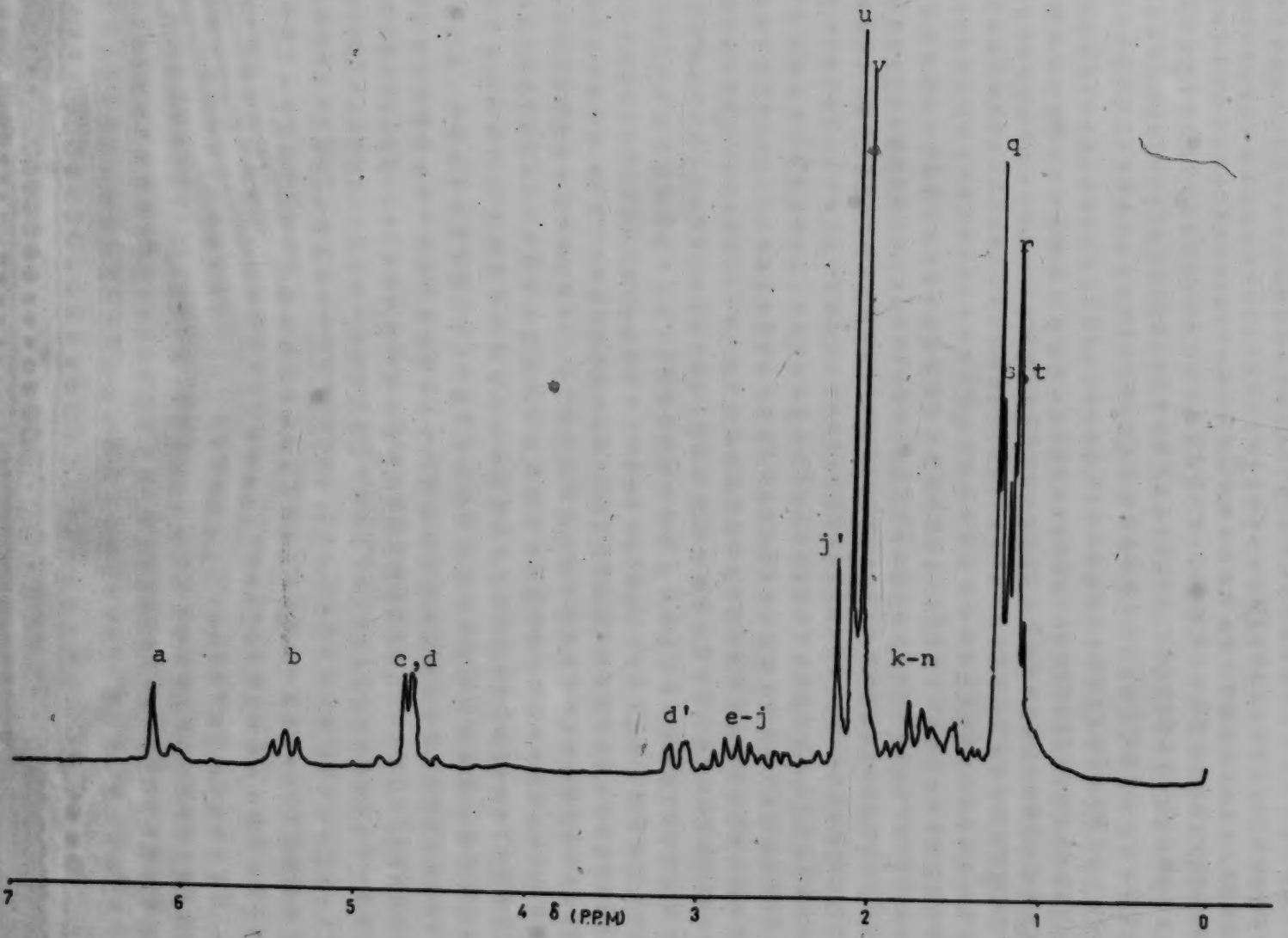

u
v
q
r
s
t
j'
a
b
c,d
d'
e-j
k-n
7
6
5
4
δ (PPM)
3
2
1
0

Table IX. Nmr data of O,O-diacetylcyafrin B_4 in $CDCl_3$

Signal	Chemical shift (δ)	Number of protons	Multiplicity	Coupling constant (Hz)
a	6.18	1	t	1
b	5.41	1	t	6
c	4.75	1	d,d	14,1
d	4.65	1	d,d	14,1
d'	3.13	1	d,d	10,1.5
e-j	3.0-2.25	about 3	u	?
j'	2.21	2	s	
k-n	1.9-1.3	about 4	u	?
q	1.28	3	s	
r	1.17	3	s	
s	1.23	3	d	7
t	1.19	3	d	7
u	2.12	3	s	
v	2.06	3	s	

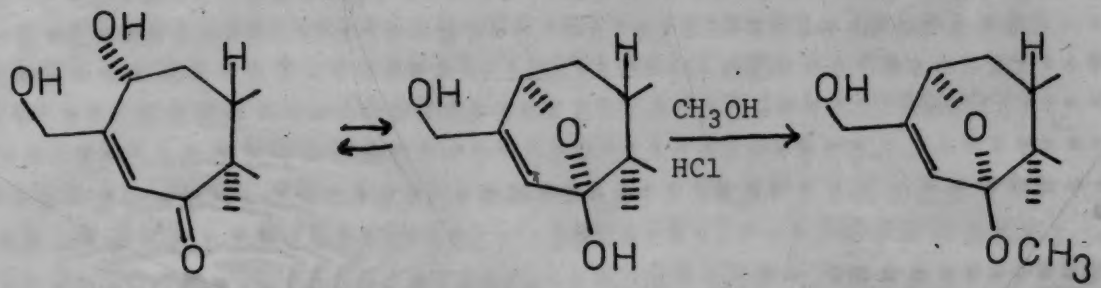
in the nmr of 0,0-diacetylcyathin A_3 are found to have shifted considerably to the low field side of the spectrum in 0,0-diacetylcyafrin B_4. From these data a few conclusions can be drawn: of four oxygen atoms present in cyafrin B_4 two exist as hydroxyl groups and a third forms part of an α,β-unsaturated ketone group; the seven-membered ring containing this α,β-unsaturated ketone and bearing the two hydroxyl groups in cyathin A_3 is present unchanged in cyafrin B_4 as evidenced by the signals a, b, c and d in the nmr spectrum of the diacetyl derivative; the molecule, like other cyathins, contains two tertiary methyl groups and an isopropyl group.

Other inferences may be made: cyafrin B_4 may have the same basic skeleton as all cyathins; the fourth oxygen atom is present in a functional group which considerably influences the chemical shifts of many protons in the molecule (signals d', q, r, s and t in the nmr spectrum are all downfield compared to their "normal" positions).

The problem is now to establish the nature of the fourth oxygen function. This oxygen atom does not exist as a third hydroxyl group; the ir spectrum of the diacetyl derivative proves this. The α,β-unsaturated ketone group present in all cyathins very often cannot be detected because it can be "masked" by virtue of the internal ketone-hemiketal equilibrium. We must therefore consider the possi-

bility of a second carbonyl group in cyafrin B_4, similarly undetected. It is also possible that the fourth oxygen is present as an ether function, e.g. as an epoxide. One of the metabolites of C. helenae, neoallocyathin A_4, has been shown to contain an epoxide function (see structure 7).

Taube[7,9] first prepared methyl ketal derivatives of cyathin A_3 and allocyathin B_3 by treatment with methanol containing hydrogen chloride, as illustrated in scheme II.



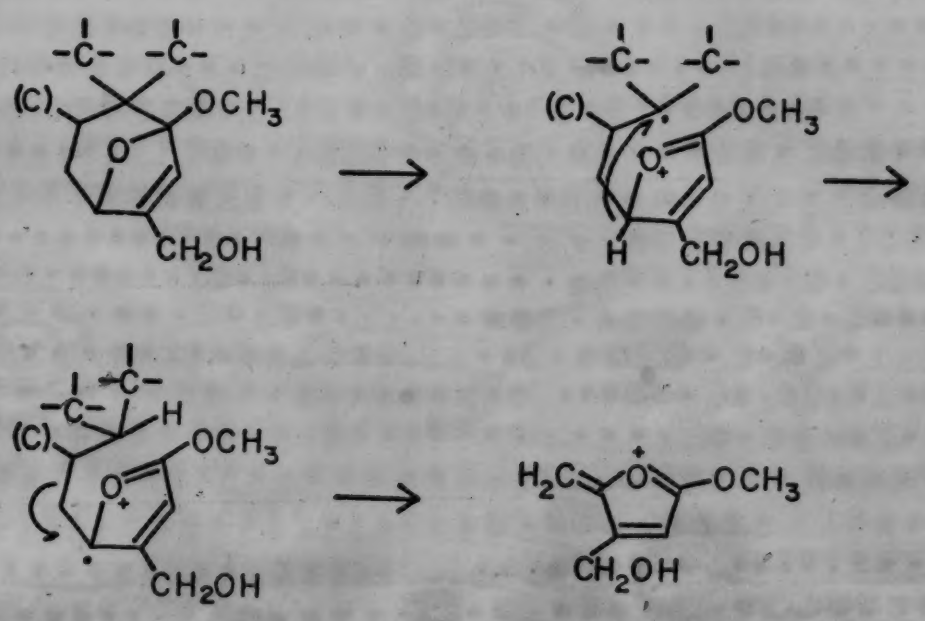
Scheme II. Preparation of methyl ketal derivatives of cyathins

The preparation of such a derivative could assist us in ascertaining whether or not cyafrin B_4 contains a second carbonyl group, since in this derivative the α,β-unsaturated ketone is ketalized. If a further carbonyl group is present, this may now be observed in the infrared

spectrum.

The methyl ketal of cyafrin B_4 was prepared by the method of Taube [7, p.132; 9]. The product was isolated by prep. tlc of the reaction mixture (solvent system A, double elution, R_f 0.4).

Mass spectroscopy (see fig. 19) indicates that the product is the desired cyafrin B_4 methyl ketal (parent peak: m/e 346, hrms: $C_{21}H_{30}O_4$). The mass spectrum shows a very intense peak at m/e 141 (hrms: $C_7H_9O_3$) which is common to methyl ketal derivatives of all cyathins. Its formation has been rationalized as illustrated in scheme III [7, p.72; 9].



Scheme III. Fragmentation of a cyathin methyl ketal

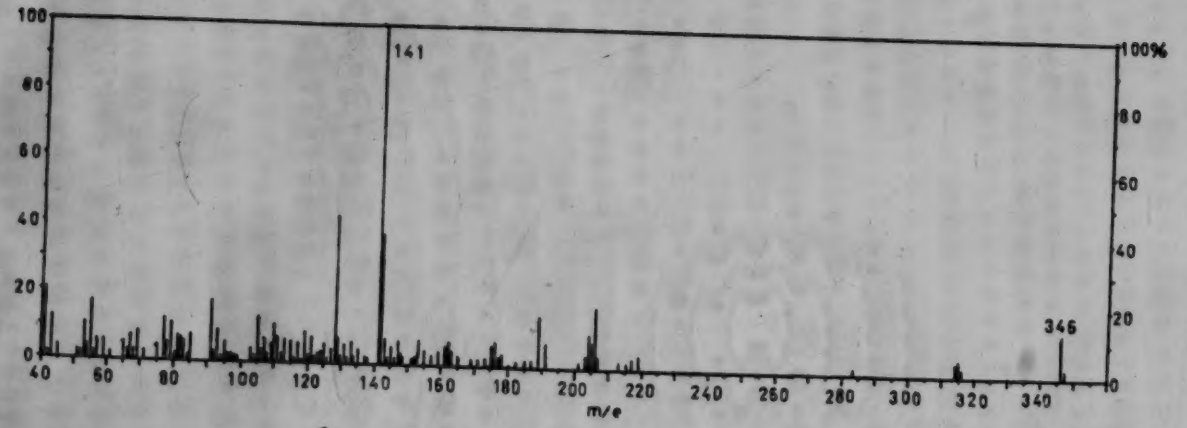


Figure 19. Mass spectrum of cyafrin B_4 methyl ketal

The ir spectrum of cyafrin B_4 methyl ketal in chloroform solution (see fig. 20) shows a strong absorption at 1685 cm^{-1} indicating the presence of a carbonyl group. Fig. 21 shows the spectrum as recorded on a neat (solid phase) sample of cyafrin B_4 methyl ketal. It is possible that the absorption found at 1685 cm^{-1} in the solution spectrum of cyafrin B_4 itself (fig. 14) is in fact due mainly to this second carbonyl group and not to the α,β-unsaturated ketone as assumed previously. Interestingly, the solution spectrum of cyafrin B_4 methyl ketal (fig. 20) shows only free hydroxyl group absorption (3600 cm^{-1}) and no hydrogen bonded hydroxyl group absorption (3400 cm^{-1}) at all, whereas the reverse is true for the solid phase ir spectrum of cyafrin B_4 methyl ketal (fig. 21).

The uv spectrum of cyafrin B_4 methyl ketal in hexane solution proves conclusively that cyafrin B_4 contains a second chromophore besides the α,β-unsaturated ketone common to all cyathins. An absorption is found at 251 nm (ε ~ 3.500), with a shoulder around 310 nm (ε ~60). This indicates that a second α,β-unsaturated ketone is present. This absorption must have been partially masked in the uv spectrum of cyafrin B_4 itself. A logarithmic plot of the uv spectrum of cyafrin B_4 reveals a shoulder around 251 nm on the peak at 231 nm (see fig. 22). Thus it has now become clear that cyafrin B_4 contains two α,β-unsaturated ketone functions.

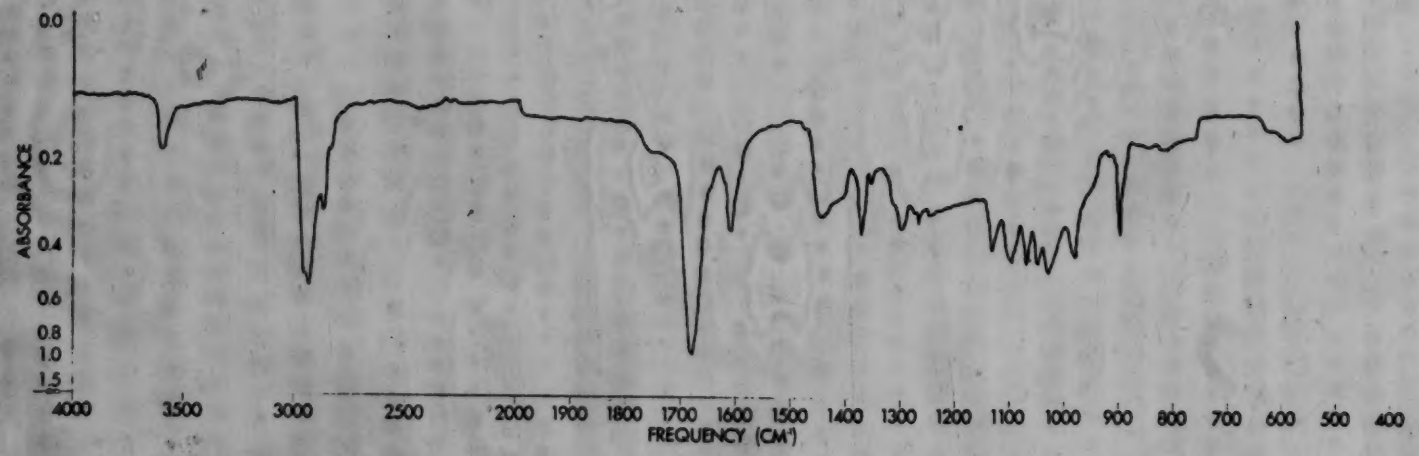


Figure 20. Ir spectrum of cyafrin B_4 methyl ketal ($CHCl_3$ solution)

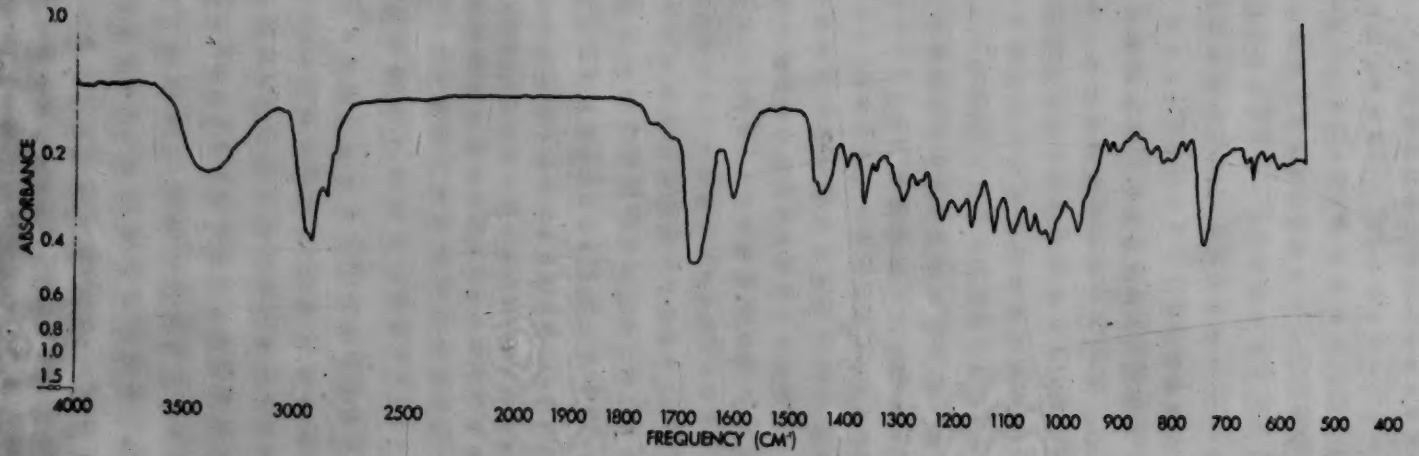


Figure 21. Ir spectrum of cyafrin B_4 methyl ketal (neat)

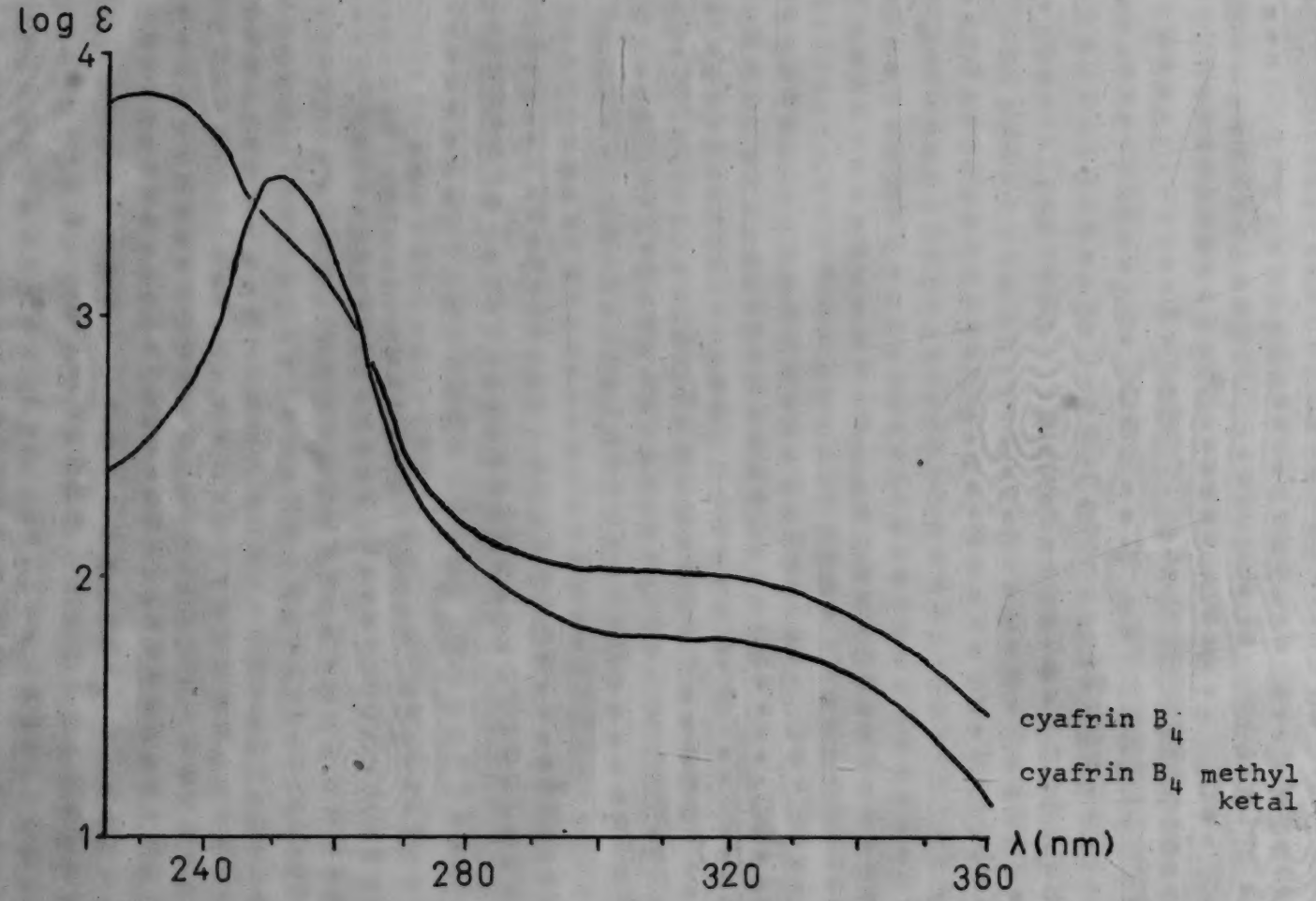
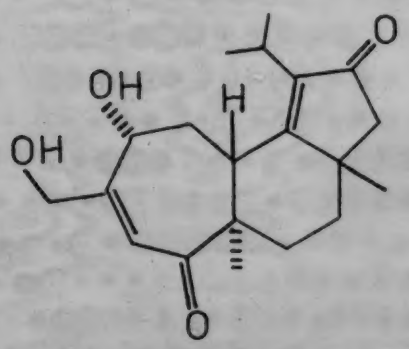
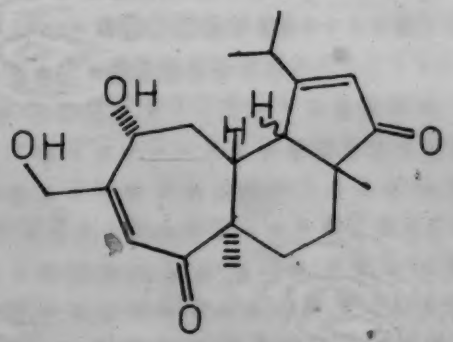


Figure 22. Uv spectra of cyafrin B_4 and cyafrin B_4 methyl ketal

If we assume cyafrin B_4 to possess a cyathin-like structure it is possible to suggest structure 21, or alternatively structure 22 for this metabolite.



21



22

The nmr spectrum of cyafrin B_4 methyl ketal (see fig. 23) allows us to choose between 21 and 22. Compare the data for cyafrin B_4 methyl ketal (table X) with the nmr data measured for cyathin A_3 methyl ketal by Taube [7, p.65; 9] (table XI).

The nmr of cyafrin B_4 methyl ketal shows (table X) signals a, b, c, d, e, q, r, s, t and z which are all virtually unchanged from their positions in the nmr spectrum of cyathin A_3 methyl ketal. All other signals of cyafrin B_4 methyl ketal have shifted to lower field, as was found in the case of 0,0-diacetylcyafrin B_4. The two proton singlet j', found at δ 2.21 in 0,0-diacetylcyafrin B_4 is

Figure 23. Nmr spectrum of cyafrin B_4 methyl ketal ($CDCl_3$)

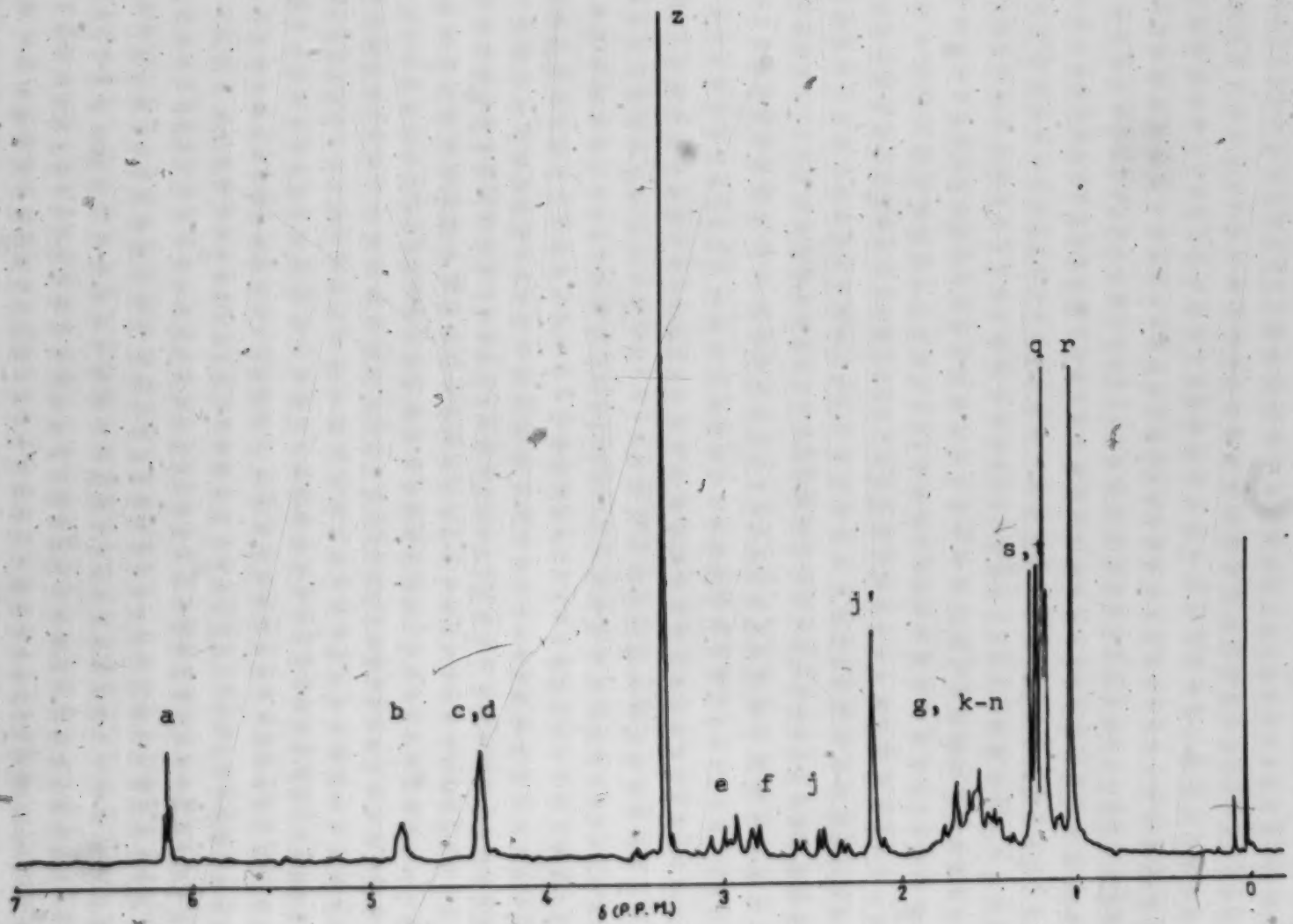

z
q r
s, t
j'
a
b
c,d
e f j
g, k-n
7
6
5
4
δ (P.P.M.)
3
2
1
0

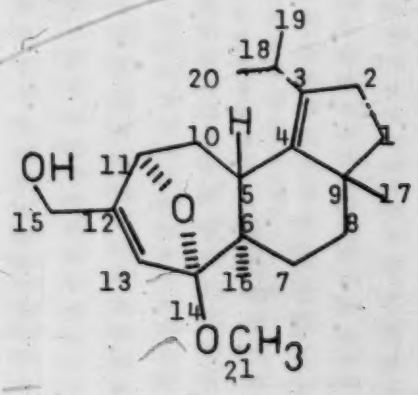
Table X. Nmr data of cyafrin B_4 methyl ketal in $CDCl_3$

Signal	Chemical shift (δ)	Number of protons	Multiplicity	Coupling constant (Hz)
a	6.15	1	t	1.5
b	4.82	1	u	?
c,d	4.38	2	u	?
e	3.00	1	q,q	7,7
f	2.89	1	d,d	12.5,4
j	2.45	1	d,d,d	12.5,12.5,3.5
j'	2.16	2	s	
g, k-n	1.8-1.2	about 5	u	?
q	1.18	3	s	
r	1.01	3	s	
s	1.22	3	d	7
t	1.19	3	d	7
z	3.34	3	s	

Table XI. Nmr data of cyathin A_3 methyl ketal in $CDCl_3$

Signal	Chemical shift (δ)	Number of protons	Multiplicity	Coupling constant (Hz)	Assignment[a]
a	6.06	1	t	1.5	13
b	4.79	1	u	?	11
c,d	4.34	2	u	?	15
e	2.95	1	q,q	6.5,6.5	18
f	around 2.42	1	d,m	12,?	5
h,i	around 2.2	2	u	?	2
j	2.25	1	d,d,d	12,12,3	10
g, k-p	1.8-1.2	7	u	?	1,7,8,10
q	1.01	3	s		(16, 17)
r	0.97	3	s		
s	1.03	3	d	6.5	(19, 20)
t	0.93	3	d	6.5	
z	3.31	3	s		21

[a] Numbers refer to numbered positions in structure 23 (cyathin A_3 methyl ketal).



23

present in cyafrin B_4 methyl ketal (table X) at δ 2.16. This establishes that signal j' does indeed belong to the compound and is not due to an impurity. The chemical shift of this signal is in accordance with the value expected for the methylene protons adjacent to a carbonyl group in an α,β-unsaturated ketone such as those on C-1 in structure 21. Signal d' in the spectrum of O,O-diacetylcyafrin B_4 (table IX) corresponds to signal f in the spectrum of cyafrin B_4 methyl ketal (table X) and cyathin A_3 methyl ketal (table XI) and may be attributed to the proton on C-5. Its position in structure 21 in relation to the α,β-unsaturated ketone explains why signal d'/f has shifted downfield compared to the values found for cyathin A_3 derivatives. The hydrogens responsible for signals h and i in cyathin A_3 methyl ketal (table XI) have of course been replaced by an oxygen atom in cyafrin B_4. Also, two protons of the unresolved multiplet around δ 1.55 in the spectrum of cyathin A_3 methyl ketal (table XI), *i.e.* those on C-1, now give rise to signal j' in the spectrum of cyafrin B_4 methyl ketal (table X).

Structure 22 can be eliminated as a possibility for cyafrin B_4 as it would not be expected to show a two proton singlet at δ 2.21 (signal j') in its nmr spectrum. It should display a signal around δ 6 due to the vinyl proton on C-2. We are thus led to propose structure 21 for

cyafrin B_4.

It should be noted here that cyafrin B_4 is not identical with the oxidation product obtained previously from cyafrin A_4 (see page 50). This provides additional evidence that in cyafrin A_4 the hydroxyl group is not located on C-2, as was concluded before on the basis of spectral evidence.

Other metabolites

Throughout this investigation it had been noted that C. africanus produces various other compounds in addition to the four major metabolites discussed thus far. These were sometimes encountered as impurities during isolations of cyafrin A_4 and B_4 and sometimes during mass spectral analysis of bands from prep. tlc which did not contain the major metabolites. Most of these were found only in very small quantities, and usually were not analyzed beyond their molecular formulas (by hrms). Below is a list of the apparent formulas of the minor compounds encountered, arranged roughly in order of increasing R_f value (in solvent system A, triple elution).

R_f 0.0-0.2: $C_{23}H_{34}O_5$, $C_{23}H_{34}O_6$, $C_{26}H_{36}O_3$, $C_{23}H_{36}O_7$, $C_{26}H_{38}O_8$, $C_{26}H_{40}O_8$.

R_f 0.2-0.4: $C_{20}H_{26}O_2$, $C_{20}H_{26}O_3$, $C_{20}H_{26}O_4$, $C_{20}H_{28}O_2$, $C_{20}H_{28}O_5$, $C_{20}H_{30}O_5$, $C_{20}H_{32}O_4$.[a]

R_f 0.4-0.6: $C_{20}H_{24}O_3$, $C_{20}H_{24}O_4$, $C_{20}H_{26}O_3$, $C_{20}H_{26}O_4$, $C_{20}H_{28}O_2$, $C_{20}H_{28}O_5$, $C_{20}H_{30}O_4$, $C_{22}H_{32}O_4$.

R_f 0.6-0.8: $C_{22}H_{30}O_5$, $C_{22}H_{32}O_5$.

R_f 0.8-1.0: $C_{21}H_{30}O_3$, $C_{21}H_{32}O_3$, $C_{30}H_{46}O$.

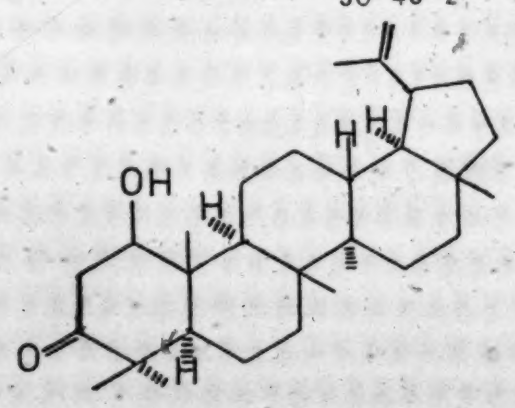
[a] This compound was encountered increasingly in samples of cyafrin B[4]. If it is isolated in pure form it will not fit conveniently into our present nomenclature system.

Of all these compounds only the ones between R_f 0.8 and 1.0 have been examined in any detail.

A sample of $C_{21}H_{30}O_3$ (containing some $C_{21}H_{32}O_3$ according to ms) was analyzed using nmr and ir; it was found that the spectral data were identical with those of allocyathin B[3] methyl ketal, as prepared by Taube[7,9], possibly contaminated with some cyathin A[3] methyl ketal. It was then realized that methanol had been used to aid dissolution of the crude cyafrin from which this compound had been isolated; apparently this treatment had been sufficient to convert some of the cyathins present into their

methyl ketal derivatives. In later separations, when use of methanol was avoided, these compounds were no longer detected.

The other compound that was studied further is the one with apparent molecular formula $C_{30}H_{46}O$. Its mass spectrum is shown in fig. 24. Exact mass determination of the fragment of m/e 229 gave as atomic composition $C_{17}H_{25}$. The ir spectrum of this compound showed, besides C-H and C-C absorption bands, a strong absorption band at 1720 cm^{-1}, which indicates the presence of an unconjugated ketone function. Uv spectroscopy showed an absorption maximum at 245 nm, but this was of such low intensity (ε ~1500) that it may well have been caused by an impurity. The total amount of this compound present in crude cyafrin was too small to allow a more detailed study at the time. Recently it has been shown[27] that this compound, the major metabolite to be found in the mycelium of C. africanus, is glochidonol (structure 24), molecular formula $C_{30}H_{48}O_2$.



24

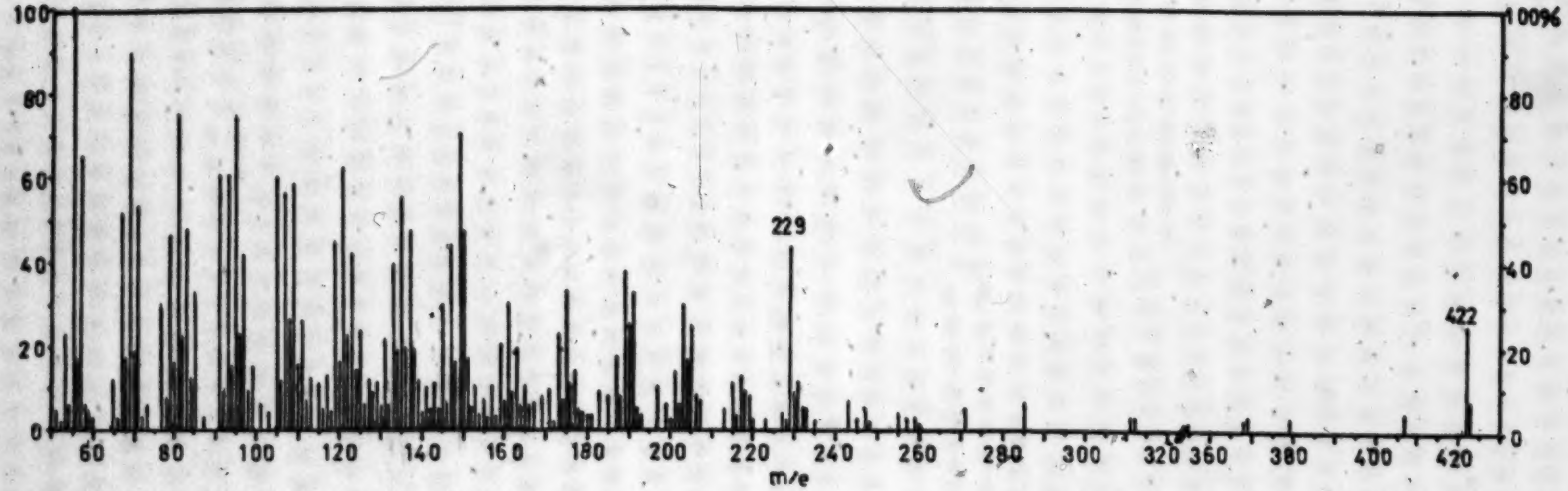


Figure 24. Mass spectrum of glochidonol

Metabolites of *Sphaerobulos stellatus*

During the screening of the metabolites of a number of species of bird's nest fungi it was found that a crude ethyl acetate extract of *S. stellatus* showed some antibacterial activity. It was decided to investigate these metabolites as well.

S. stellatus was cultured under the same conditions as described for *C. africanus*. Crude ethyl acetate extracts were obtained as described for *C. africanus*. In addition, the extracted culture was made basic to pH 10 with dilute sodium hydroxide and extracted with methylene chloride to obtain any basic components.

The total amount of crude material isolated from *S. stellatus* per liter of medium extracted was rather low (only about 30 to 50 mg per liter) compared to the 75 to 150 mg per liter we obtained from *C. africanus*.

The crude basic extracts were examined by tlc (solvent system C) and found to contain mainly highly polar material (R_f 0.0) and one small spot at R_f 0.1. A very small quantity of this latter material was isolated by prep. tlc (solvent system C). Mass spectroscopy indicated a molecular weight of 168, apparent molecular formula $C_{11}H_8N_2$. It was not possible to obtain a good ir spectrum

on this small amount of material. Surprisingly for a compound that appears to be highly unsaturated, we could not detect any significant absorption in the uv spectrum over 220 nm. Later cultures of the fungus did not produce this compound, and it was not further investigated.

Preliminary investigation by tlc (solvent system C) of the crude ethyl acetate extracts revealed a complex mixture, most of it with a low R_f value, but also containing some less polar material. Partial separation of this mixture was achieved by means of a distribution between equal volumes of methanol (containing 5% water) and Skellysolve B (a low boiling mixture of hydrocarbons). This resulted in a large "polar fraction" and a much smaller "non-polar fraction".

The "non-polar fraction" was examined by mass spectroscopy and uv. It was found to contain mainly dioctylphthalate, a commonly used plasticizer which is readily recognizable from a very prominent peak in the mass spectrum at m/e 149 and other peaks at m/e 167 and 279. No other compounds could be isolated in amounts sufficient for investigation.

The much larger "polar fraction" was subjected to tlc examination using various solvent systems, with a view to separating it by prep. tlc. However, none of the

systems used appeared to be capable of separating the constituents. Usually a streak of material was found on tlc rather than separated spots.

An attempt was made to achieve some separation of this mixture by column chromatography, using silicic acid as adsorbent, and eluting with chloroform - methanol mixtures containing an increasing percentage of methanol (up to 20%). Numerous fractions were collected, and those containing visible amounts of material after evaporation of the solvent were subjected to mass spectral analysis. Peaks were found at m/e 386, 368, 260, 170 and 103. However, during an attempt to obtain the atomic composition of these peaks only the peak at m/e 103 was reproduced. Its molecular composition was found to be $C_4H_7O_3$. The total amount of material isolated in this way was very small; the bulk of the material could only be recovered from the column by eluting with pure methanol.

It thus appeared that most of the material was highly polar. It was considered that acetylation of this polar mixture would result in reduction of its polarity and thus perhaps facilitate separation. Also this would facilitate the use of gas chromatography (gc) as an analytical and preparative method.

Acetylation of the mixture was achieved in the

usual way, using a large excess of pyridine - acetic anhydride overnight at room temperature. The reagents were removed by adding toluene and distilling the mixed solvents under reduced pressure, repeating this several times until the residue appeared to be free of the reagents.

The acetylated mixture was used for trial separations using gas chromatography (gc). The best separation was obtained using an OV-225 column (3% on chromosorb W) of 7' x $\frac{1}{4}$" and temperature programming (100^{o}-220^{o}C at 2^{o}C per minute). In this way as many as thirteen peaks could be detected (fid detector) in the mixture, one of which (at a short retention time) was by far the largest.

Preparative gc was first investigated as a method of isolating this compound. However, it was found that material collected in this way, when re-examined by analytical gc, no longer contained this component; apparently it decomposes during gas chromatography.

Prep. tlc was next investigated, separation being monitored by isolation of separated bands and then analysis of the mixtures obtained from each band by gc. Of several solvent systems tried none gave a clean separation.

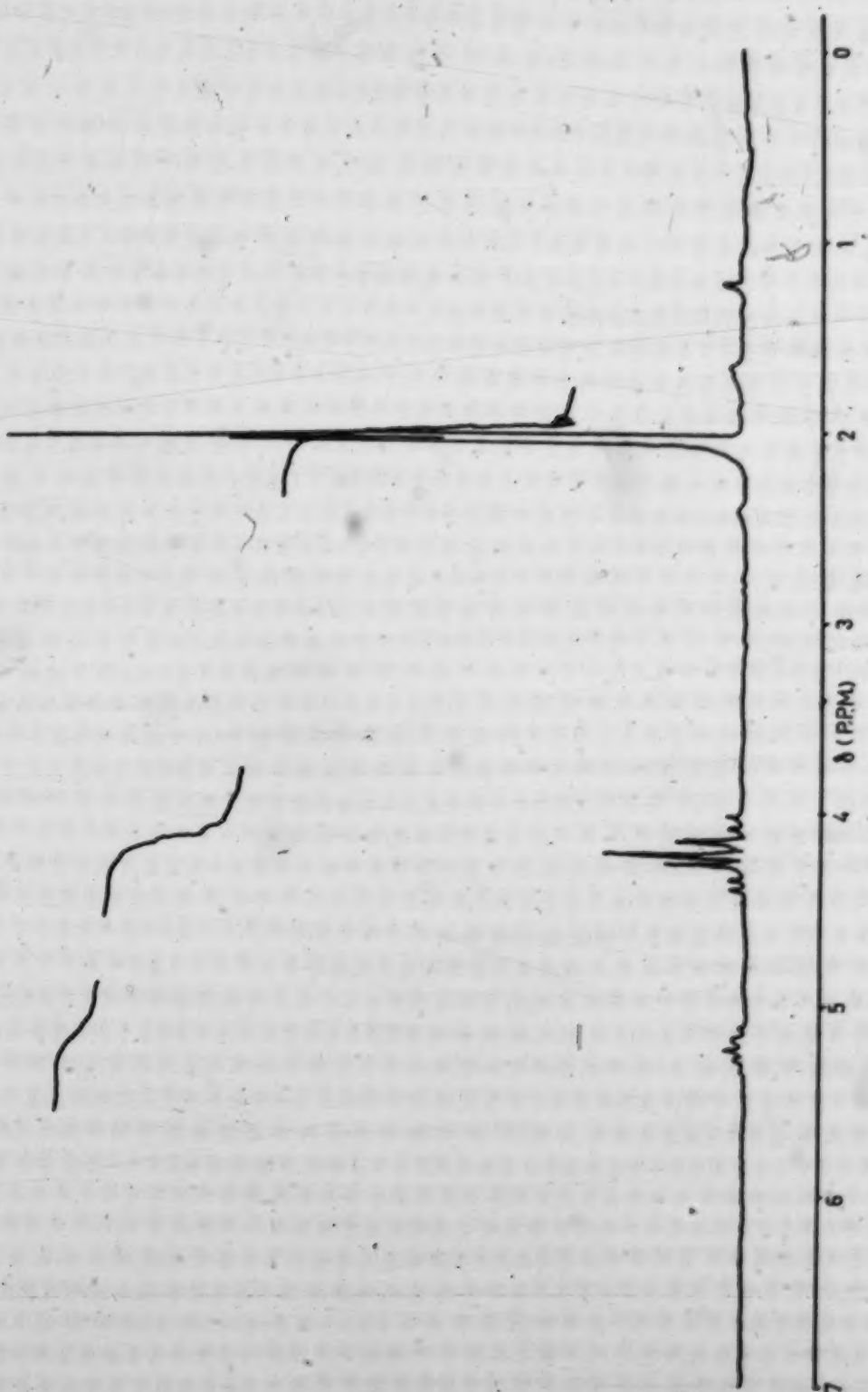
Finally the major component was isolated by column chromatography, using silica gel G as adsorbent and

eluting it with pure chloroform containing approximately 0.75% ethanol as stabilizer. Under these conditions it was the first compound eluted.

The mass spectrum of the compound shows peaks at m/e 158, 145, 116, 115, 103, 87 and 43. Aside from the base peak at m/e 43, the strongest peak is the one at m/e 103 which has an atomic composition of $C_4H_7O_3$. The nmr spectrum (see fig. 25) is very simple; a double peak at δ2.05, integrated at nine protons, a four proton triplet at δ 4.20 and a one proton quintet at δ 5.22. The ir spectrum showed absorption peaks at 955, 1020, 1060, 1090, 1265, 1370, 1420 and 1745 cm^{-1}. No uv absorption maxima were found above 210 nm..

The spectra proved to be identical with those of triacetylglycerol. Glycerol is one of the components of the Brodie medium used for growing S. stellatus. It thus appeared that the most abundant compound in the crude extracts was not a metabolite produced by the fungus. Since minor compounds were not amenable to separation and since we had no indication of the presence of cyathin-like compounds, the extracts were not examined further.

Figure 28. Nmr spectrum of triacetylglycerol ($CDCl_3$)


0
1
2
3
4
δ (P.P.M.)
5
6
7

IV. DETAILED EXPERIMENTAL

General remarks

The experimental data given in this chapter are in many cases examples of experiments which were carried out several times. The amounts used and the yields varied.

Spectral data presented in chapter III are not repeated, but reference is made to the page where they are recorded.

Growth of fungi

The procedures for growing the fungi that were investigated are described under General Experimental. The Brodie medium used contains per liter: glycerine, 6 ml; peptone, 0.2 g; d,l-asparagine, 0.2 g; yeast extract, 2.0 g; $MgSO_4$ (anhydrous), 0.24 g; $Ca(NO_3)_2.4H_2O$, 0.5 g; KH_2PO_4, 0.5 g; $Fe_2(SO_4)_3$, trace; maltose, 5.0 g; dextrose, 2.0 g. For liquid cultures this was made up to one liter with distilled water. For solid media 15 g bacto-agar was added, and distilled water to make up one liter.

The procedure used for preparing crude extracts is also given under General Experimental.

Isolation of crude metabolites

Crude cyafrin was freed from highly polar material by the ether - water partition procedure described previously. The resulting partially purified cyafrin was dissolved in a small volume of methylene chloride and chromatographed on prep. tlc plates of 100 cm length, applying 0.5 g cyafrin to each plate. The plates were developed with solventsystem A (triple elution). Three bands, at R_f 0.2, 0.4 and 0.5 were removed from the plates and eluted to yield, after evaporation of the solvent on a rotary evaporator, crude cyafrin A_4, crude cyafrin B_4 and crude cyathin A_3 / allocyathin B_3 mixture respectively.

Acetylation and purification of the crude cyathin A_3 / allocyathin B_3 mixture

Crude cyathin A_3 / allocyathin B_3 (50 mg) was dissolved in 2 ml of pyridine. Acetic anhydride (1 ml) was added and the mixture was allowed to stand overnight at room temperature. Toluene (15 ml) was added and the mixed solvents were removed by evaporation under reduced pressure using a rotary evaporator. This toluene treatment was repeated three times. The residue was taken up in methylene chloride and chromatographed on one 20 x 20 cm prep. tlc plate (solvent system A). A band at R_f 0.7 was removed from the plate and eluted to yield, after evaporation of the solvent, 15 mg of

a 3 : 4 mixture of 0,0-diacetylcyathin A_3 and 0,0-diacetylallocyathin B_3. The mass spectrum of this mixture is shown in fig. 1. The nmr spectrum is given in fig. 2.

Purification of cyafrin A_4 and cyafrin B_4

Crude cyafrin A_4 (200 mg, isolated from several growths of fungus) was dissolved in a small amount of acetone and applied to two 20 x 20 cm prep. tlc plates. These were developed with solvent system D. The band at R_f 0.6 was removed from the plates and eluted with acetone. After evaporation of the solvent 140 mg of purified cyafrin A_4 was obtained. The mass spectrum of this compound is shown in fig. 3. Ir spectra are shown in fig. 5 and 6. The nmr spectrum is shown in fig. 4.

Crude cyafrin B_4 (300 mg, isolated from several growths) was dissolved in a small amount of acetone and chromatographed on three 20 x 20 cm. prep. tlc plates with solvent system C, quadruple elution. The band at R_f 0.4, after elution with acetone and evaporation of the solvent, yielded 94 mg purified cyafrin B_4. The mass spectrum of this compound is shown in fig. 13. Ir spectra are shown in fig. 14 and 15. The uv spectrum is shown in fig. 22.

Acetylation of cyafrin A_4 and cyafrin B_4

Crude cyafrin A_4 (75 mg) was acetylated using the procedure outlined for cyathin A_3 / allocyathin B_3. The excess reagents were removed by repeated co-evaporation under reduced pressure with toluene. The residue was chromatographed on a 20 x 20 cm prep. tlc plate using solvent system A. A band found at R_f 0.65 yielded pure 0,0,0-triacetylcyafrin A_4 (45 mg). The mass spectrum of this compound is shown in fig. 7. The nmr data are given in fig. 8 and tables III and VIII.

Cyafrin B_4 (30 mg) was acetylated in the same manner. After removal of the excess reagents prep. tlc (solvent system A) yielded 0,0-diacetylcyafrin B_4 (21 mg). Mass spectral analysis of this material showed it to be impure. Further purification was achieved by prep. tlc (solvent system B, double elution) on a 5 x 20 cm plate. A band at R_f 0.5 yielded pure 0,0-diacetylcyafrin B_4 (8 mg). The mass spectrum of this compound is shown in fig. 16. The ir spectrum is shown in fig. 17. Nmr data are given in fig. 18 and table IX.

Formation of the acetonide of cyafrin A_4

Cyafrin A_4 (15 mg) was dissolved in 2,2-dimethoxypropane (1 ml) to which a few drops of acetone had been added to assist dissolution. A few small crystals of p-to-

luenesulphonic acid were added. After 30 minutes the reaction mixture was applied directly to a 5 x 20 cm prep. tlc plate, which was developed with solvent system A. The band at R_f 0.5 yielded pure 0,0-isopropylidenecyafrin A_4 (9 mg). The mass spectrum of this compound is shown in fig. 9. Nmr data are given in fig. 10 and tables IV and VI.

"Jones" oxidation of 0,0-isopropylidenecyafrin A_4

0,0-isopropylidenecyafrin A_4 (5 mg) was dissolved in acetone (0.2 ml). "Jones reagent" (8N CrO_3 in dilute sulphuric acid, 15 microliter) was added. The orange colour of the reagent changed to green. A small drop of 2-propanol was added to consume excess reagent and a small amount of Na_2CO_3 was added to neutralize the solution. The solids were filtered off and the reaction mixture was applied to a 5 x 20 cm prep. tlc plate. After development with solvent system A, the band at R_f 0.75 yielded dehydrocyafrin A_4 acetonide (approx. 1 mg). For analytical data on this compound see page 50.

Attempted dehydration of 0,0-isopropylidenecyafrin A_4

0,0-isopropylidenecyafrin A_4 (5 mg) and collidine (0.3 ml) were dissolved in a small amount of methylene chloride. Alumina (Woelm neutral alumina - grade 1, 0.5 g) was added, and the methylene chloride removed by evaporation.

The resulting dry powder was heated at 160°C for 30 minutes, then extracted first with 10 ml methylene chloride and then with 10 ml of methanol. The combined extracts were concentrated by evaporation. Tlc examination of the residue (solvent system A) showed no spots with R_f greater than 0.0. No starting material could be detected. This material was not further characterized.

Formation of 1-chlorocyathin A_3 acetonide

0,0-isopropylidenecyafrin A_4 (7 mg) was dissolved in dry acetone (0.5 ml). Pyridine (20 microliter) was added and the solution was cooled to 0°C in an ice-water bath, then 10 microliter of freshly distilled thionyl chloride was added. After 50 minutes the reaction mixture was applied directly to a 5 x 20 cm prep. tlc plate, which was developed with solvent system A. The band at R_f 0.8 yielded what is believed to be 1-chlorocyathin A_3 acetonide (2 mg). The mass spectrum of this compound is shown in fig. 11. The nmr spectrum of this compound is shown in fig. 12.

Formation of methyl ketal of cyafrin B_4

Cyafrin B_4 (90 mg, impure) was dissolved in 1.5 ml of a 2.5 N solution of HCl in methanol. After 4 hours the reaction mixture was applied to a 20 x 20 cm prep. tlc plate. After development with solvent system A (double elu-

tion) a band at R_f 0.4 gave cyafrin B_4 methyl ketal (45 mg). The mass spectrum of this compound is shown in fig. 19. The ir spectra are shown in fig. 20 and 21. The uv spectrum is shown in fig. 22. Nmr data are given in fig. 23 and table X.

Isolation of triacetylglycerol

Some of the methanol soluble fraction of the ethyl acetate extract from S. stellatus was acetylated as described for cyathin A_3 / allocyathin B_3. About 100 mg of this acetylated crude was chromatographed on a column of silica gel G (E. Merck), 1 cm diameter, 10 cm high. The column was eluted with chloroform (containing approx. 0.75% ethanol as stabilizer). The fractions which were collected were analyzed by glc, under conditions described on page 97. The first five fractions yielded, after evaporation of the solvent, glycerol triacetate (33 mg). The nmr spectrum of this compound is shown in fig. 28.

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